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(54) Title: FUSION PROTEINS FOR USE IN ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES (57) Abstract <p>This invention provides fusion polypeptides that include a glycosyltransferase catalytic domain and a catalytic domain from an accessory enzyme that is involved in making a substrate for a glycosyltransferase reaction. Nucleic acids that encode the fusion polypeptides are also provided, as are host cells for expressing the fusion polypeptides of the invention.</p>		

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FUSION PROTEINS FOR USE IN ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES

5 CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of, and claims benefit of, US Provisional Application No. 60/069,443, filed December 15, 1997, which application is incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

10 Field of the Invention

This invention pertains to the field of enzymatic synthesis of oligosaccharides using fusion proteins that can catalyze more than one reaction involved in the enzymatic synthesis.

Background

15 Increased understanding of the role of carbohydrates as recognition elements on the surface of cells has led to increased interest in the production of carbohydrate molecules of defined structure. For instance, compounds comprising the sialyl Lewis ligands, sialyl Lewis^x and sialyl Lewis^a are present in leukocyte and non-leukocyte cell lines that bind to receptors such as the ELAM-1 and GMP 140 receptors. Polley *et al.*, *Proc. Natl. Acad. Sci. USA* (1991) 88: 6224 and Phillips *et al.* (1990) *Science* 250: 1130, *see, also*, US
20 Patent No. 5,753,631.

Because of interest in making desired carbohydrate structures, glycosyltransferases and their role in enzyme-catalyzed synthesis of carbohydrates are presently being extensively studied. These enzymes exhibit high specificity and are useful
25 in forming carbohydrate structures of defined sequence. Consequently, glycosyltransferases are increasingly used as enzymatic catalysts in synthesis of a number of carbohydrates used for therapeutic and other purposes. In the application of enzymes to the field of synthetic carbohydrate chemistry, the use of glycosyltransferases for enzymatic synthesis of carbohydrate offers advantages over chemical methods due to the virtually complete

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stereoselectivity and linkage specificity offered by the enzymes (Ito *et al.* (1993) *Pure Appl. Chem.* 65: 753; and U.S. Patents 5,352,670, and 5,374,541).

Chemoenzymatic syntheses of oligosaccharides and of corresponding derivatives therefore represent an interesting opportunity to develop novel therapeutic agents. However this approach is still hampered by the relatively poor availability of the required glycosyltransferases and the difficulty and cost of obtaining substrates for these enzymes. Large-scale enzymatic syntheses of oligosaccharides will also require large amounts of the accessory enzymes necessary for the synthesis of the sugar-nucleotides that are used as the donors by the glycosyltransferases. The present invention provides fusion proteins that simplify the purification of enzymes that are useful for enzymatic synthesis of oligosaccharides.

SUMMARY OF THE INVENTION

The present invention provides fusion polypeptides that are useful for enzymatic synthesis of oligosaccharides. The fusion polypeptides of the invention have a catalytic domain of a glycosyltransferase joined to a catalytic domain of an accessory enzyme. The accessory enzyme catalytic domain can, for example, catalyze a step in the formation of a nucleotide sugar which is a donor for the glycosyltransferase, or catalyze a reaction involved in a glycosyltransferase cycle.

In another embodiment, the invention provides nucleic acids that include a polynucleotide that encodes a fusion polypeptide. The fusion polypeptides have a catalytic domain of a glycosyltransferase, and a catalytic domain of an accessory enzyme. Expression cassettes and expression vectors that include the nucleic acids are also provided, as are host cells that contain the nucleic acids of the invention.

The invention also provides methods of producing a fusion polypeptide that has a catalytic domain of a glycosyltransferase and a catalytic domain of an accessory enzyme. The methods involve introducing a nucleic acid that encodes the fusion polypeptide into a host cell to produce a transformed host cell; and culturing the transformed host cell under conditions appropriate for expressing the fusion polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of recombinant sialyltransferase/CMP-NeuAc synthetase fusion protein of the invention. The C terminus of the CMP-Neu5Ac synthetase is linked covalently to the N terminus of the α -2,3-sialyltransferase through a 9-residue peptide linker. The first Met residue of the α -2,3-sialyltransferase was replaced by a Leu residue (underlined in the linker sequence). The C terminus of the fusion protein also includes a c-Myc epitope tag for immuno-detection and a His₆ tail for purification by IMAC. The total length of the fusion protein encoded by pFUS-01/2 is 625 residues.

Figure 2 shows the nucleotide (SEQ ID NO: 1) and deduced amino acid (SEQ ID NO: 2) sequences of *lgtB* from *Neisseria meningitidis*.

Figure 3 shows a diagram of a recombinant fusion protein that catalyzes transfer of galactose residues from a donor to an acceptor. The C terminus of the UDP-Glc/Gal epimerase is linked covalently to the N terminus of the β -1,4-Galactosyltransferase through a 4-residue peptide linker. The first Met residue of the β -1,4-Galactosyltransferase was replaced by a Val residue (underlined in the linker sequence). The total length of the fusion protein encoded by pFUS-EB is 611 residues.

Figure 4 shows primers that were used in the construction of the UDP-Glc/Gal epimerase/ β -1,4-Galactosyltransferase fusion protein.

DETAILED DESCRIPTION

Definitions

The fusion proteins of the invention are useful for transferring a monosaccharide from a donor substrate to an acceptor molecule, and/or for forming a reactant that is involved in the saccharide transfer reaction. The addition generally takes place at the non-reducing end of an oligosaccharide or carbohydrate moiety on a biomolecule. Biomolecules as defined here include but are not limited to biologically significant molecules such as carbohydrates, proteins (e.g., glycoproteins), and lipids (e.g., glycolipids, phospholipids, sphingolipids and gangliosides).

The following abbreviations are used herein:

Ara = arabinosyl;
Fru = fructosyl;
Fuc = fucosyl;

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Gal = galactosyl;

GalNAc= N-acetylgalactosylamino;

Glc = glucosyl;

GlcNAc= N-acetylglucosylamino;

5 Man = mannosyl; and

NeuAc = sialyl (N-acetylneuraminy).

Oligosaccharides are considered to have a reducing end and a non-reducing end, whether or not the saccharide at the reducing end is in fact a reducing sugar. In accordance with accepted nomenclature, oligosaccharides are depicted herein with the non-reducing end on the left and the reducing end on the right.

All oligosaccharides described herein are described with the name or abbreviation for the non-reducing saccharide (*e.g.*, Gal), followed by the configuration of the glycosidic bond (α or β), the ring bond, the ring position of the reducing saccharide involved in the bond, and then the name or abbreviation of the reducing saccharide (*e.g.*, GlcNAc). The linkage between two sugars may be expressed, for example, as 2,3, 2 \rightarrow 3, or (2,3). Each saccharide is a pyranose or furanose.

Donor substrates for glycosyltransferases are activated nucleotide sugars. Such activated sugars generally consist of uridine, guanosine, and cytidine monophosphate or diphosphate derivatives of the sugars in which the nucleoside monophosphate or diphosphate serves as a leaving group. The donor substrate for sialyltransferases, for example, are activated sugar nucleotides comprising the desired sialic acid. For instance, in the case of NeuAc, the activated sugar is CMP-NeuAc.

The term "sialic acid" refers to 5-N-acetylneuraminic acid (NeuAc) or 5-N-glycolylneuraminic acid (NeuGc), as well as other sialic acids may be used in their place, however. For a review of different forms of sialic acid suitable in the present invention see, Schauer, *Methods in Enzymology*, 50: 64-89 (1987), and Schaur, *Advances in Carbohydrate Chemistry and Biochemistry*, 40: 131-234.

A "fusion glycosyltransferase polypeptide" of the invention is glycosyltransferase fusion polypeptide that contains a glycosyltransferase catalytic domain and a second catalytic domain from an accessory enzyme (*e.g.*, a CMP-Neu5Ac synthetase or a UDP-Glucose 4' epimerase (*galE*)) and is capable of catalyzing the transfer of an

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oligosaccharide residue from a donor substrate (e.g., CMP-NeuAc or UDP-Gal) to an acceptor molecule. Typically, such polypeptides will be substantially similar to the exemplified proteins disclosed here.

An "accessory enzyme," as referred to herein, is an enzyme that is involved in catalyzing a reaction that, for example, forms a substrate for a glycosyltransferase. An accessory enzyme can, for example, catalyze the formation of a nucleotide sugar that is used as a donor moiety by a glycosyltransferase. An accessory enzyme can also be one that is used in the generation of a nucleotide triphosphate required for formation of a nucleotide sugar, or in the generation of the sugar which is incorporated into the nucleotide sugar.

A "catalytic domain" refers to a portion of an enzyme that is sufficient to catalyze an enzymatic reaction that is normally carried out by the enzyme. For example, a catalytic domain of a sialyltransferase will include a sufficient portion of the sialyltransferase to transfer a sialic acid residue from a donor to an acceptor saccharide. A catalytic domain can include an entire enzyme, a subsequence thereof, or can include additional amino acid sequences that are not attached to the enzyme or subsequence as found in nature.

Much of the nomenclature and general laboratory procedures required in this application can be found in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989. The manual is hereinafter referred to as "Sambrook *et al.*"

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the second sequence.

A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same

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source, is modified from its original form. Thus, a heterologous glycosyltransferase gene in a particular host cell includes a glycosyltransferase gene that is endogenous to the particular host cell but has been modified. Modification of the heterologous nucleic acid can occur, *e.g.*, by treating the DNA with a restriction enzyme to generate a DNA fragment that is
5 capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

A "subsequence" refers to a sequence of nucleic acids or amino acids that comprise a part of a longer sequence of nucleic acids or amino acids (*e.g.*, polypeptide) respectively.

10 The term "recombinant" when used with reference to a cell indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Recombinant cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell
15 by artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques.

A "recombinant expression cassette" or simply an "expression cassette" is a
20 nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of affecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a desired polypeptide), and a promoter.
25 Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include nucleotide sequences that encode a signal sequence that directs secretion of an expressed protein from the host cell. Transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

30 The term "isolated" is meant to refer to material which is substantially or essentially free from components which normally accompany the material as found in its

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native state. Thus, an isolated material does not include materials normally associated with their *in situ* environment. Typically, isolated proteins of the invention are at least about 80% pure, usually at least about 90%, and preferably at least about 95% pure as measured by band intensity on a silver stained gel or other method for determining purity. Protein purity or
5 homogeneity can be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualization upon staining. For certain purposes high resolution will be needed and HPLC or a similar means for purification utilized.

The terms "identical" or percent "identity," in the context of two or more
10 nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or
15 polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more
20 preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison
25 algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by
30 the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the

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search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally, Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between
5 Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)).

Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are
10 described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which
15 either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be
20 increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved
25 value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid
30 sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E)

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of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

The phrase "hybridizing specifically to", refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

The term "stringent conditions" refers to conditions under which a probe will hybridize to its target subsequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 15 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at T_m , 50% of the probes are occupied at equilibrium). Typically, stringent conditions will be those in which the salt concentration is less than about

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1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as
5 formamide.

The phrases "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies
10 bind preferentially to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to
15 select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

"Conservatively modified variations" of a particular polynucleotide sequence
20 refers to those polynucleotides that encode identical or essentially identical amino acid sequences, or where the polynucleotide does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine.
25 Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every polynucleotide sequence described herein which encodes a polypeptide also describes every possible silent variation, except where otherwise noted.
30 One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and UGG which is ordinarily the only codon for

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tryptophan) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

One of skill will appreciate that many conservative variations of the fusion proteins and nucleic acid which encode the fusion proteins yield essentially identical products. For example, due to the degeneracy of the genetic code, "silent substitutions" (*i.e.*, substitutions of a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of every nucleic acid sequence which encodes an amino acid. As described herein, sequences are preferably optimized for expression in a particular host cell used to produce the chimeric endonucleases (*e.g.*, yeast, human, and the like). Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties (*see*, the definitions section, *supra*), are also readily identified as being highly similar to a particular amino acid sequence, or to a particular nucleic acid sequence which encodes an amino acid. Such conservatively substituted variations of any particular sequence are a feature of the present invention. *See also*, Creighton (1984) *Proteins*, W.H. Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations".

Description of the Preferred Embodiments

The present invention provides fusion polypeptides that include a glycosyltransferase catalytic domain and at least one catalytic domain of one or more accessory enzymes. Accessory enzymes can, for example, catalyze a step in the formation of a nucleotide sugar which is a donor for the glycosyltransferase. Nucleic acids that encode the

fusion polypeptides are also provided, as are expression vectors and host cells that include these nucleic acids.

The fusion polypeptides of the invention find use in the enzymatic synthesis of oligosaccharides. Significant advantages are provided by the fusion polypeptides. For example, the use of a fusion polypeptide that has two or more enzymatic activities reduces the number of polypeptides that must be obtained for a given synthesis. Thus, purification is simplified.

A. *Glycosyltransferases*

The fusion polypeptides of the invention include a catalytic domain of a glycosyltransferase. The catalytic domain can be from any of a wide variety of glycosyltransferases. Among the glycosyltransferases from one which one can obtain a catalytic domain are the sialyltransferases, *N*-acetylglucosaminyltransferases, *N*-acetylgalactosaminyltransferases, fucosyltransferases, galactosyltransferases, glucosyltransferases, xylosyltransferases, and mannosyltransferases.

The glycosyltransferases can be either prokaryotic or eukaryotic glycosyltransferases.

Eukaryotic glycosyltransferases

The fusion polypeptides of the present invention can include a catalytic domain of a eukaryotic glycosyltransferase. Eukaryotic glycosyltransferases typically have topological domains at their amino terminus that are not required for catalytic activity (*see*, US Patent No. 5, 032,519). The "cytoplasmic domain," which is most commonly between about 1 and about 10 amino acids in length, is the most amino-terminal domain. The adjacent domain, termed the "signal-anchor domain," is generally between about 10-26 amino acids in length. Adjacent to the signal-anchor domain is a "stem region," which is typically between about 20 and about 60 amino acids in length. The stem region functions as a retention signal to maintain the glycosyltransferase in the Golgi apparatus. The catalytic domain of the glycosyltransferase is found to the carboxyl side of the stem region.

In a presently preferred embodiment, the glycosyltransferase catalytic domains that are present in the fusion proteins of the invention substantially lack one or more of the cytoplasmic, signal-anchor, and stem region domains. More preferably, two of

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these domains are at least substantially absent from the fusion protein, and most preferably all three of the cytoplasmic domain, the signal-anchor domain, and the stem region are substantially or completely absent from the fusion proteins of the invention.

Many mammalian glycosyltransferases have been cloned and expressed and the recombinant proteins have been characterized in terms of donor and acceptor specificity and they have also been investigated through site directed mutagenesis in attempts to define residues involved in either donor or acceptor specificity (Aoki *et al.* (1990) *EMBO. J.* 9: 3171-3178; Harduin-Lepers *et al.* (1995) *Glycobiology* 5(8): 741-758; Natsuka and Lowe (1994) *Current Opinion in Structural Biology* 4: 683-691; Zu *et al.* (1995) *Biochem. Biophys. Res. Comm.* 206(1): 362-369; Seto *et al.* (1995) *Eur. J. Biochem.* 234: 323-328; Seto *et al.* (1997) *J. Biol. Chem.* 272: 14133-14138).

In some embodiments, the glycosyltransferase catalytic domain is obtained from a fucosyltransferase. A number of fucosyltransferases are known to those of skill in the art. Briefly, fucosyltransferases include any of those enzymes which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. In some embodiments, for example, the acceptor sugar is a GlcNAc in a Gal β (1 \rightarrow 4)GlcNAc group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the known Gal β (1 \rightarrow 3,4)GlcNAc α (1 \rightarrow 3,4)fucosyltransferase (FTIII, E.C. No. 2.4.1.65) which is obtained from human milk (*see, Palcic, et al., Carbohydrate Res.* 190:1-11 (1989); Prieels, *et al., J. Biol. Chem.* 256: 10456-10463 (1981); and Nunez, *et al., Can. J. Chem.* 59: 2086-2095 (1981)) and the Gal β (1 \rightarrow 4)GlcNAc α (1 \rightarrow 3)fucosyltransferases (FTIV, FTV, FTVI, and FTVII, E.C. No. 2.4.1.65) which are found in human serum. A recombinant form of Gal β (1 \rightarrow 3,4)GlcNAc α (1 \rightarrow 3,4)fucosyltransferase is also available (*see, Dumas, et al., Bioorg. Med. Letters* 1:425-428 (1991) and Kukowska-Latallo, *et al., Genes and Development* 4:1288-1303 (1990)). Other exemplary fucosyltransferases include α 1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, *et al., Eur. J. Biochem.* 191:169-176 (1990) or U.S. Patent No. 5,374,655.

In another group of embodiments, the glycosyltransferase catalytic domain is obtained from a galactosyltransferase. Exemplary galactosyltransferases include α 1,3-galactosyltransferases (E.C. No. 2.4.1.151, *see, e.g., Dabkowski et al., Transplant Proc.* 25:2921 (1993) and Yamamoto *et al. Nature* 345:229-233 (1990), bovine (GenBank j04989,

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Joziassse *et al.* (1989) *J. Biol. Chem.* 264:14290-14297), murine (GenBank m26925; Larsen *et al.* (1989) *Proc. Nat'l. Acad. Sci. USA* 86:8227-8231), porcine (GenBank L36152; Strahan *et al.* (1995) *Immunogenetics* 41:101-105)). Another suitable α 1,3-galactosyltransferase is that which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto *et al.* (1990) *J. Biol. Chem.* 265:1146-1151 (human)). Also suitable for use in the fusion polypeptides of the invention are α 1,4-galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al.* (1989) *Eur. J. Biochem.* 183:211-217), human (Masri *et al.* (1988) *Biochem. Biophys. Res. Commun.* 157:657-663), murine (Nakazawa *et al.* (1988) *J. Biochem.* 104:165-168), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al.* (1994) *J. Neurosci. Res.* 38:234-242). Other suitable galactosyltransferases include, for example, α 1,2-galactosyltransferases (from *e.g.*, *Schizosaccharomyces pombe*, Chapell *et al.* (1994) *Mol. Biol. Cell* 5:519-528).

Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (preferably a rat ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al.* (1996) *Glycobiology* 6: v-xiv). An exemplary α 2,3-sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal β 1 \rightarrow 4GlcNAc disaccharide or glycoside. See, Van den Eijnden *et al.*, *J. Biol. Chem.*, 256:3159 (1981), Weinstein *et al.*, *J. Biol. Chem.*, 257:13845 (1982) and Wen *et al.*, *J. Biol. Chem.*, 267:21011 (1992). Another exemplary α 2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of a Gal β 1 \rightarrow 3GalNAc disaccharide or glycoside. See, Rearick *et al.*, *J. Biol. Chem.*, 254: 4444 (1979) and Gillespie *et al.*, *J. Biol. Chem.*, 267:21004 (1992). Further exemplary enzymes include Gal- β -1,4-GlcNAc α -2,6 sialyltransferase (See, Kurosawa *et al.* *Eur. J. Biochem.* 219: 375-381 (1994)). Sialyltransferase nomenclature is described in Tsuji, S. *et al.* (1996) *Glycobiology* 6:v-vii.

Other glycosyltransferases that can be used in the fusion polypeptides of the invention have been described in detail, as for the sialyltransferases, galactosyltransferases, and fucosyltransferases. In particular, the glycosyltransferase can also be, for instance,

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glucosyltransferases, e.g., Alg8 (Stagljov *et al.*, *Proc. Natl. Acad. Sci. USA* 91:5977 (1994)) or Alg5 (Heesen *et al.* *Eur. J. Biochem.* 224:71 (1994)), N-acetylgalactosaminyltransferases such as, for example, $\beta(1,3)$ -N-acetylgalactosaminyltransferase; $\beta(1,4)$ -N-acetylgalactosaminyltransferases (US Patent No. 5,691,180, Nagata *et al.* *J. Biol. Chem.* 267:12082-12089 (1992), and Smith *et al.* *J. Biol. Chem.* 269:15162 (1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa *et al.* *J. Biol. Chem.* 268:12609 (1993)). Suitable N-acetylglucosaminyltransferases include GnTI (2.4.1.101, Hull *et al.*, *BBRC* 176:608 (1991)), GnTII, and GnTIII (Ihara *et al.* *J. Biochem.* 113:692 (1993)), GnTV (Shoreiban *et al.* *J. Biol. Chem.* 268: 15381 (1993)), O-linked N-acetylglucosaminyltransferase (Bierhuizen *et al.* *Proc. Natl. Acad. Sci. USA* 89:9326 (1992)), N-acetylglucosamine-1-phosphate transferase (Rajput *et al.* *Biochem J.* 285:985 (1992), and hyaluronan synthase. Also of interest are enzymes involved in proteoglycan synthesis, such as, for example, N-acetylgalactosaminyltransferase I (EC 2.4.1.174), and enzymes involved in chondroitin sulfate synthesis, such as N-acetylgalactosaminyltransferase II (EC 2.4.1.175). Suitable mannosyltransferases include $\alpha(1,2)$ mannosyltransferase, $\alpha(1,3)$ mannosyltransferase, $\beta(1,4)$ mannosyltransferase, Dol-P-Man synthase, OCh1, and Pmt1. Xylosyltransferases include, for example, protein xylosyltransferase (EC 2.4.2.26).

Prokaryotic glycosyltransferases

In other embodiments, the fusion proteins of the invention include a glycosyltransferase catalytic domain from a prokaryotic glycosyltransferase. Nucleic acids encoding several prokaryotic glycosyltransferases have been cloned and characterized, and can be used in the fusion proteins of the invention. As is the case for eukaryotic glycosyltransferases, prokaryotic glycosyltransferases often have a membrane-spanning domain near the amino terminus that can be omitted, if desired, from the fusion polypeptide. Suitable prokaryotic glycosyltransferases include enzymes involved in synthesis of lipooligosaccharides (LOS), which are produced by many Gram negative bacteria. The LOS typically have terminal glycan sequences that mimic glycoconjugates found on the surface of human epithelial cells or in host secretions (Preston *et al.* (1996) *Critical Reviews in Microbiology* 23(3): 139-180). Such enzymes include, but are not limited to, the proteins of the *rfa* operons of species such as *E. coli* and *Salmonella typhimurium*, which include a $\alpha 1,6$ -galactosyltransferase and a $\alpha 1,3$ -galactosyltransferase (*see, e.g.*,

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EMBL Accession Nos. M80599 and M86935 (*E. coli*); EMBL Accession No. S56361 (*S. typhimurium*)), a glucosyltransferase (Swiss-Prot Accession No. P25740 (*E. coli*), an α 1,2-glucosyltransferase (*rfaJ*)(Swiss-Prot Accession No. P27129 (*E. coli*) and Swiss-Prot Accession No. P19817 (*S. typhimurium*)), and an α 1,2-*N*-acetylglucosaminyltransferase (*rfaK*)(EMBL Accession No. U00039 (*E. coli*). Other glycosyltransferases for which amino acid and/or nucleic acid sequences are known include those that are encoded by operons such as *rfaB*, which have been characterized in organisms such as *Klebsiella pneumoniae*, *E. coli*, *Salmonella typhimurium*, *Salmonella enterica*, *Yersinia enterocolitica*, *Mycobacterium leprosum*, and the *rhl* operon of *Pseudomonas aeruginosa*.

Also suitable for use in the fusion proteins of the invention are glycosyltransferases that are involved in producing structures containing lacto-*N*-neotetraose, D-galactosyl- β -1,4-*N*-acetyl-D-glucosaminyl- β -1,3-D-galactosyl- β -1,4-D-glucose, and the P^k blood group trisaccharide sequence, D-galactosyl- α -1,4-D-galactosyl- β -1,4-D-glucose, which have been identified in the LOS of the mucosal pathogens *Neisseria gonorrhoeae* and *N. meningitidis* (Scholten *et al.* (1994) *J. Med. Microbiol.* 41: 236-243). The genes from *N. meningitidis* and *N. gonorrhoeae* that encode the glycosyltransferases involved in the biosynthesis of these structures have been identified from *N. meningitidis* immunotypes L3 and L1 (Jennings *et al.* (1995) *Mol. Microbiol.* 18: 729-740) and the *N. gonorrhoeae* mutant F62 (Gotshlich (1994) *J. Exp. Med.* 180: 2181-2190). In *N. meningitidis*, a locus consisting of 3 genes, *lgtA*, *lgtB* and *lgtE*, encodes the glycosyltransferase enzymes required for addition of the last three of the sugars in the lacto-*N*-neotetraose chain (Wakarchuk *et al.* (1996) *J. Biol. Chem.* 271: 19166-73). Recently the enzymatic activity of the *lgtB* and *lgtA* gene product was demonstrated, providing the first direct evidence for their proposed glycosyltransferase function (Wakarchuk *et al.* (1996) *J. Biol. Chem.* 271 (45): 28271-276). In *N. gonorrhoeae*, there are two additional genes, *lgtD* which adds β -D-GalNAc to the 3 position of the terminal galactose of the lacto-*N*-neotetraose structure and *lgtC* which adds a terminal α -D-Gal to the lactose element of a truncated LOS, thus creating the P^k blood group antigen structure (Gotshlich (1994), *supra.*). In *N. meningitidis*, a separate immunotype L1 also expresses the P^k blood group antigen and has been shown to carry an *lgtC* gene (Jennings *et al.* (1995), *supra.*). *Neisseria* glycosyltransferases and associated genes are also described in USPN 5,545,553

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(Gotschlich). An α 1,3-fucosyltransferase gene from *Helicobacter pylori* has also been characterized (Martin *et al.* (1997) *J. Biol. Chem.* 272: 21349-21356).

Sialyltransferases from prokaryotes have been described by, for example, Weisgerber *et al.* (1991) *Glycobiol.* 1:357-365; Frosch, M. *et al.* (1991) *Mol. Microbiol.* 5:1251-1263; and Gilbert, M. *et al.* (1996) *J. Biol. Chem.* 271:28271-28276. It has been suggested that the bacterial sialyltransferases might have a wider spectrum of acceptors than their mammalian counterparts (Kajihara, Y. *et al.* (1996) *J. Org. Chem.* 61:8632-8635 and Gilbert *et al.*, *Eur. J. Biochem.* 249: 187-194 (1997)).

As is the case for eukaryotic glycosyltransferases, one can readily obtain nucleic acids that encode other prokaryotic glycosyltransferases that can be used in constructing fusion polypeptides according to the invention.

B. Accessory enzymes involved in nucleotide sugar formation

The fusion polypeptides of the invention include, in addition to the glycosyltransferase catalytic domain(s), at least one catalytic domain from an accessory enzyme. Accessory enzymes include, for example, those enzymes that are involved in the formation of a nucleotide sugar. The accessory enzyme can be involved in attaching the sugar to a nucleotide, or can be involved in making the sugar or the nucleotide, for example. The nucleotide sugar is generally one that is utilized as a saccharide donor by the glycosyltransferase catalytic domain of the particular fusion polypeptide. Examples of nucleotide sugars that are used as sugar donors by glycosyltransferases include, for example, GDP-Man, UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, CMP-sialic acid, UDP-xylose, GDP-Fuc, GDP-GlcNAc, among others.

Accessory enzymes that are involved in synthesis of nucleotide sugars are well known to those of skill in the art. For a review of bacterial polysaccharide synthesis and gene nomenclature, *see, e.g.*, Reeves *et al.*, *Trends Microbiol.* 4: 495-503 (1996). The methods described above for obtaining glycosyltransferase-encoding nucleic acids are also applicable to obtaining nucleic acids that encode enzymes involved in the formation of nucleotide sugars. For example, one can use one of nucleic acids known in the art, some of which are listed below, directly or as a probe to isolate a corresponding nucleic acid from other organisms of interest.

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As one example, to produce a galactosylated soluble oligosaccharide, a galactosyltransferase is often used. However, galactosyltransferases generally use as a galactose donor the activated nucleotide sugar UDP-Gal, which is comparatively expensive. To reduce the expense of the reaction, one can construct one or more fusion polypeptides that have the galactosyltransferase catalytic domain and also a catalytic domain from one of the accessory enzymes that are involved in the biosynthetic pathway which leads to UDP-Gal. For example, glucokinase (EC 2.7.1.12) catalyzes the phosphorylation of glucose to form Glc-6-P. Genes that encode glucokinase have been characterized (*e.g.*, *E. coli*: GenBank AE000497 U00096, Blattner *et al.*, *Science* 277: 1453-1474 (1997); *Bacillus subtilis*: GenBank Z99124, AL009126, Kunst *et al.*, *Nature* 390, 249-256 (1997)), and thus can be readily obtained from many organisms by, for example, hybridization or amplification. A fusion polypeptide that contains a catalytic domain from this enzyme, as well as those of the subsequent enzymes in the pathway as set forth below, will thus be able to form UDP-glucose from readily available glucose, which can be either produced by the organism or added to the reaction mixture.

The next step in the pathway leading to UDP-Gal is catalyzed by phosphoglucomutase (EC 5.4.2.2), which converts Glc-6-P to Glc-1-P. Again, genes encoding this enzyme have been characterized for a wide range of organisms (*e.g.*, *Agrobacterium tumefaciens*: GenBank AF033856, Uttaro *et al.* *Gene* 150: 117-122 (1994) [published erratum appears in *Gene* (1995) 155:141-3]; *Entamoeba histolytica*: GenBank Y14444, Ortner *et al.*, *Mol. Biochem. Parasitol.* 90, 121-129 (1997); *Mesembryanthemum crystallinum*: GenBank U84888; *S. cerevisiae*: GenBank X72016, U09499, X74823, Boles *et al.*, *Eur. J. Biochem.* 220: 83-96 (1994), Fu *et al.*, *J. Bacteriol.* 177 (11), 3087-3094 (1995); human: GenBank M83088 (PGM1), Whitehouse *et al.*, *Proc. Nat'l. Acad. Sci. U.S.A.* 89: 411-415 (1992), *Xanthomonas campestris*: GenBank M83231, Koeplin *et al.*, *J. Bacteriol.* 174: 191-199 (1992); *Acetobacter xylinum*: GenBank L24077, Brautaset *et al.*, *Microbiology* 140 (Pt 5), 1183-1188 (1994); *Neisseria meningitidis*: GenBank U02490, Zhou *et al.*, *J. Biol. Chem.* 269 (15), 11162-11169 (1994).

UDP-glucose pyrophosphorylase (EC 2.7.7.9) catalyzes the next step in the pathway, conversion of Glc-1-P to UDP-Glc. Genes encoding UDP-Glc pyrophosphorylase are described for many organisms (*e.g.*, *E. coli*: GenBank M98830, Weissborn *et al.*, *J.*

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Bacteriol. 176: 2611-2618 (1994); *Cricetulus griseus*: GenBank AF004368, Flores-Diaz *et al.*, *J. Biol. Chem.* 272: 23784-23791 (1997); *Acetobacter xylinum*: GenBank M76548, Brede *et al.*, *J. Bacteriol.* 173, 7042-7045 (1991); *Pseudomonas aeruginosa* (*galU*): GenBank AJ010734, U03751; *Streptococcus pneumoniae*: GenBank AJ004869; *Bacillus subtilis*: GenBank Z22516, L12272; Soldo *et al.*, *J. Gen. Microbiol.* 139 (Pt 12), 3185-3195 (1993); *Solanum tuberosum*: GenBank U20345, L77092, L77094, L77095, L77096, L77098, U59182, Katsube *et al.*, *J. Biochem.* 108: 321-326 (1990); *Hordeum vulgare* (barley): GenBank X91347; *Shigella flexneri*: GenBank L32811, Sandlin *et al.*, *Infect. Immun.* 63: 229-237 (1995); human: GenBank U27460, Duggleby *et al.*, *Eur. J. Biochem.* 235 (1-2), 173-179 (1996); bovine: GenBank L14019, Konishi *et al.*, *J. Biochem.* 114, 61-68 (1993).

Finally, UDP-Glc 4'-epimerase (UDP-Gal 4' epimerase; EC 5.1.3.2) catalyzes the conversion of UDP-Glc to UDP-Gal. The *Streptococcus thermophilus* UDPgalactose 4-epimerase gene described by Poolman *et al.* (*J. Bacteriol.* 172: 4037-4047 (1990)) is a particular example of a gene that is useful in the present invention. Exemplary genes encoding UDPglucose 4-epimerase include those of *E. coli*, *K. pneumoniae*, *S. lividans*, and *E. stewartii*, as well as *Salmonella* and *Streptococcus* species. Nucleotide sequences are known for UDP-Glc 4'-epimerases from several organisms, including *Pasteurella haemolytica*, GenBank U39043, Potter *et al.*, *Infect. Immun.* 64 (3), 855-860 (1996); *Yersinia enterocolitica*, GenBank Z47767, X63827, Skurnik *et al.*, *Mol. Microbiol.* 17: 575-594 (1995); *Cyamopsis tetragonoloba*: GenBank AJ005082; *Pachysolen tannophilus*: GenBank X68593, Skrzypek *et al.*, *Gene* 140 (1), 127-129 (1994); *Azospirillum brasilense*: GenBank Z25478, De Troch *et al.*, *Gene* 144 (1), 143-144 (1994); *Arabidopsis thaliana*: GenBank Z54214, Dormann *et al.*, *Arch. Biochem. Biophys.* 327: 27-34 (1996); *Bacillus subtilis*: GenBank X99339, Schrogel *et al.*, *FEMS Microbiol. Lett.* 145: 341-348 (1996); *Rhizobium meliloti*: GenBank X58126 S81948, Buendia *et al.*, *Mol. Biol.* 5: 1519-1530 (1991); *Rhizobium leguminosarum*: GenBank X96507; *Erwinia amylovora*: GenBank X76172, Metzger *et al.*, *J. Bacteriol.* 176: 450-459 (1994); *S. cerevisiae*: GenBank X81324 (cluster of epimerase and UDP-glucose pyrophosphorylase), Schaaff-Gerstenschlager, *Yeast* 11: 79-83 (1995); *Neisseria meningitidis*: GenBank U19895, L20495, Lee *et al.*, *Infect.*

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Immun. 63: 2508-2515 (1995), Jennings *et al.*, *Mol. Microbiol.* 10: 361-369 (1993); and *Pisum sativum*: GenBank U31544.

Often, genes encoding enzymes that make up a pathway involved in synthesizing nucleotide sugars are found in a single operon or region of chromosomal DNA.

5 For example, the *Xanthomonas campestris* phosphoglucomutase, phosphomannomutase, (xanA), phosphomannose isomerase, and GDP-mannose pyrophosphorylase (xanB) genes are found on a single contiguous nucleic acid fragment (Koeplin *et al.*, *J. Bacteriol.* 174, 191-199 (1992)). *Klebsiella pneumoniae* galactokinase, galactose-1-phosphate uridylyltransferase, and UDP-galactose 4'-epimerase are also found in a single operon (Peng *et*

10 *al.* (1992) *J. Biochem.* 112: 604-608). Many other examples are described in the references cited herein.

An alternative galactosyltransferase fusion polypeptide can include a catalytic domain from UDP-Gal pyrophosphorylase (galactose-1-phosphate uridylyltransferase), which converts Gal-1-P to UDP-Gal. Genes that encode UDP-Gal pyrophosphorylase have been

15 characterized for several organisms, including, for example, *Rattus norvegicus*: GenBank L05541, Heidenreich *et al.*, *DNA Seq.* 3: 311-318 (1993); *Lactobacillus casei*: GenBank AF005933 (cluster of galactokinase (*galK*), UDP-galactose 4-epimerase (*galE*), galactose 1-phosphate-uridylyltransferase (*galT*)), Bettenbrock *et al.*, *Appl. Environ. Microbiol.* 64: 2013-2019 (1998); *E. coli*: GenBank X06226 (*galE* and *galT* for UDP-galactose-4-epimerase and

20 galactose-1-P uridylyltransferase), Lemaire *et al.*, *Nucleic Acids Res.* 14: 7705-7711 (1986)); *B. subtilis*: GenBank Z99123 AL009126; *Neisseria gonorrhoeae*: GenBank Z50023, Ulrich *et al.*, *J. Bacteriol.* 177: 6902-6909 (1995); *Haemophilus influenzae*: GenBank X65934 (cluster of galactose-1-phosphate uridylyltransferase, galactokinase, mutarotase and galactose repressor), Maskell *et al.*, *Mol. Microbiol.* 6: 3051-3063 (1992), GenBank M12348 and

25 M12999, Tajima *et al.*, *Yeast* 1: 67-77 (1985)); *S. cerevisiae*: GenBank X81324, Schaaff-Gerstenschlager *et al.*, *Yeast* 11: 79-83 (1995); *Mus musculus*: GenBank U41282; human: GenBank M96264, M18731, Leslie *et al.*, *Genomics* 14: 474-480 (1992), Reichardt *et al.*, *Mol. Biol. Med.* 5: 107-122 (1988); *Streptomyces lividans*: M18953 (galactose 1-phosphate uridylyltransferase, UDP-galactose 4-epimerase, and galactokinase), Adams *et al.*, *J.*

30 *Bacteriol.* 170: 203-212 (1988).

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Catalytic domains of UDP-GlcNAc 4' epimerase (UDP-GalNAc 4'-epimerase)(EC 5.1.3.7), which catalyzes the conversion of UDP-GlcNAc to UDP-GalNAc, and the reverse reaction, are also suitable for use in the fusion polypeptides of the invention. Several loci that encode this enzyme are described above. *See also*, US Patent No.

5 5,516,665.

Another example of a fusion polypeptide provided by the invention is used for producing a fucosylated soluble oligosaccharide. The donor nucleotide sugar for fucosyltransferases is GDP-fucose, which is relatively expensive to produce. To reduce the cost of producing the fucosylated oligosaccharide, the invention provides fusion
10 polypeptides that can convert the relatively inexpensive GDP-mannose into GDP-fucose, and then catalyze the transfer of the fucose to an acceptor saccharide. These fusion polypeptides include a catalytic domain from at least one of a GDP-mannose dehydratase, a GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase, or a GDP-4-keto-6-deoxy-L-glucose 4-reductase. When each of these enzyme activities is provided, one can convert GDP-mannose
15 into GDP-fucose.

The nucleotide sequence of an *E. coli* gene cluster that encodes GDP-fucose-synthesizing enzymes is described by Stevenson *et al.* (1996) *J. Bacteriol.* 178: 4885-4893; GenBank Accession No. U38473). This gene cluster had been reported to include an open reading frame for GDP-mannose dehydratase (nucleotides 8633-9754; Stevenson *et al.*,
20 *supra.*). It was recently discovered that this gene cluster also contains an open reading frame that encodes an enzyme that has both 3,5 epimerization and 4-reductase activities (*see*, commonly assigned US Provisional Patent Application No. 60/071,076, filed January 15, 1998), and thus is capable of converting the product of the GDP-mannose dehydratase reaction (GDP-4-keto-6-deoxymannose) to GDP-fucose. This ORF, which is designated
25 YEF B, is found between nucleotides 9757-10722. Prior to this discovery that YEF B encodes an enzyme having two activities, it was not known whether one or two enzymes were required for conversion of GDP-4-keto-6-deoxymannose to GDP-fucose. The nucleotide sequence of a gene encoding the human Fx enzyme is found in GenBank Accession No. U58766.

30 Also provided are fusion polypeptides that include a mannosyltransferase catalytic domain and a catalytic domain of a GDP-Man pyrophosphorylase (EC 2.7.7.22),

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which converts Man-1-P to GDP-Man. Suitable genes are known from many organisms, including *E. coli*: GenBank U13629, AB010294, D43637 D13231, Bastin *et al.*, *Gene* 164: 17-23 (1995), Sugiyama *et al.*, *J. Bacteriol.* 180: 2775-2778 (1998), Sugiyama *et al.*, *Microbiology* 140 (Pt 1): 59-71 (1994), Kido *et al.*, *J. Bacteriol.* 177: 2178-2187 (1995);
5 *Klebsiella pneumoniae*: GenBank AB010296, AB010295, Sugiyama *et al.*, *J. Bacteriol.* 180: 2775-2778 (1998); *Salmonella enterica*: GenBank X56793 M29713, Stevenson *et al.*, *J. Bacteriol.* 178: 4885-4893 (1996).

The fusion polypeptides of the invention for fucosylating a saccharide acceptor can also utilize enzymes that provide a minor or "scavenge" pathway for GDP-
10 fucose formation. In this pathway, free fucose is phosphorylated by fucokinase to form fucose 1-phosphate, which, along with guanosine 5'-triphosphate (GTP), is used by GDP-fucose pyrophosphorylase to form GDP-fucose (Ginsburg *et al.*, *J. Biol. Chem.*, 236: 2389-2393 (1961) and Reitman, *J. Biol. Chem.*, 255: 9900-9906 (1980)). Accordingly, a fucosyltransferase catalytic domain can be linked to a catalytic domain from a GDP-fucose
15 pyrophosphorylase, for which suitable nucleic acids are described in copending, commonly assigned US Patent Application Ser. No. 08/826,964, filed April 9, 1997. Fucokinase-encoding nucleic acids are described for, *e.g.*, *Haemophilus influenzae* (Fleischmann *et al.* (1995) *Science* 269:496-512) and *E. coli* (Lu and Lin (1989) *Nucleic Acids Res.* 17: 4883-4884).

20 Other pyrophosphorylases are known that convert a sugar phosphate into a nucleotide sugar. For example, UDP-GalNAc pyrophosphorylase catalyzes the conversion of GalNAc to UDP-GalNAc. UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23) converts GlcNAc-1-P to UDP-GlcNAc (*B. subtilis*: GenBank Z99104 AL009126, Kunst *et al.*, *supra.*; *Candida albicans*: GenBank AB011003, Mio *et al.*, *J. Biol. Chem.* 273 (23), 14392-14397 (1998);
25 *Saccharomyces cerevisiae*: GenBank AB011272, Mio *et al.*, *supra.*; human: GenBank AB011004, Mio *et al.*, *supra.*). These can also be used in the fusion polypeptides of the invention.

The invention also provides fusion polypeptides that are useful for sialylation reactions. These fusion polypeptides include a catalytic domain from a sialyltransferase and
30 a catalytic domain from a CMP-sialic acid synthetase (EC 2.7.7.43, CMP-N-acetylneuraminic acid synthetase). Such genes are available from, for example, *Mus*

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musculus (GenBank AJ006215, Munster *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95: 9140-9145 (1998)), rat (Rodríguez-Aparicio *et al.* (1992) *J. Biol. Chem.* 267: 9257-63), *Haemophilus ducreyi* (Tullius *et al.* (1996) *J. Biol. Chem.* 271: 15373-80), *Neisseria meningitidis* (Ganguli *et al.* (1994) *J. Bacteriol.* 176: 4583-9), group B streptococci (Haft *et al.* (1994) *J. Bacteriol.* 176: 7372-4), and *E. coli* (GenBank J05023, Zapata *et al.* (1989) *J. Biol. Chem.* 264: 14769-14774). Alternatively, fusion proteins for sialylation reactions can have a catalytic domain from either or both of GlcNAc 2' epimerase (EC 5.1.3.8), which converts GlcNAc to ManNAc, and neuraminic acid aldolase (EC 4.1.3.3; SwissProt Accession No. P06995), which in turn converts the ManNAc to sialic acid.

Additional accessory enzymes from which one can obtain a catalytic domain are those that are involved in forming reactants consumed in a glycosyltransferase cycle. For example, any of several phosphate kinases are useful as accessory enzymes. Polyphosphate kinase (EC 2.7.4.1), for example, catalyzes the formation of ATP; nucleoside phosphate kinases (EC 2.7.4.4) can form the respective nucleoside diphosphates; creatine phosphate kinase (EC 2.7.3.2); myokinase (EC 2.7.4.3); *N*-acetylglucosamine acetyl kinase (EC 2.7.1.59); acetyl phosphate kinase; and pyruvate kinase (EC 2.7.1.40).

C. Cloning of glycosyltransferase and accessory enzyme nucleic acids

Nucleic acids that encode glycosyltransferases and accessory enzymes, and methods of obtaining such nucleic acids, are known to those of skill in the art. Suitable nucleic acids (*e.g.*, cDNA, genomic, or subsequences (probes)) can be cloned, or amplified by *in vitro* methods such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (SSR). A wide variety of cloning and *in vitro* amplification methodologies are well-known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook *et al.*); *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing

Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel); Cashion *et al.*, U.S. patent number 5,017,478; and Carr, European Patent No. 0,246,864.

DNA that encodes glycosyltransferase and accessory enzyme polypeptides, or subsequences thereof, can be prepared by any suitable method described above, including, for example, cloning and restriction of appropriate sequences. In one preferred embodiment, a nucleic acid encoding a glycosyltransferase or accessory enzyme can be isolated by routine cloning methods. A nucleotide sequence of a glycosyltransferase or accessory enzyme as provided in, for example, GenBank or other sequence database (see above) can be used to provide probes that specifically hybridize to a glycosyltransferase or accessory enzyme gene in a genomic DNA sample, or to a glycosyltransferase or accessory enzyme mRNA in a total RNA sample (*e.g.*, in a Southern or Northern blot). Once the target glycosyltransferase or accessory enzyme nucleic acid is identified, it can be isolated according to standard methods known to those of skill in the art (*see, e.g.*, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory; Berger and Kimmel (1987) *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques*, San Diego: Academic Press, Inc.; or Ausubel *et al.* (1987) *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York). Alternatively, subsequences can be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

A glycosyltransferase nucleic acid can also be cloned by detecting its expressed product by means of assays based on the physical, chemical, or immunological properties. For example, one can identify a cloned glycosyltransferase nucleic acid by the ability of a polypeptide encoded by the nucleic acid to catalyze the transfer of a monosaccharide from a donor to an acceptor moiety. In a preferred method, capillary electrophoresis is employed to detect the reaction products. This highly sensitive assay involves using either monosaccharide or disaccharide aminophenyl derivatives which are labeled with fluorescein as described in Wakarchuk *et al.* (1996) *J. Biol. Chem.* 271 (45): 28271-276. For example, to assay for a *Neisseria lgtC* enzyme, either FCHASE-AP-Lac or FCHASE-AP-Gal can be used, whereas for the *Neisseria lgtB* enzyme an appropriate reagent is FCHASE-AP-GlcNAc (*Id.*).

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As an alternative to cloning a glycosyltransferase or accessory enzyme gene or cDNA, a glycosyltransferase nucleic acid can be chemically synthesized from a known sequence that encodes a glycosyltransferase. Suitable methods include the phosphotriester method of Narang *et al.* (1979) *Meth. Enzymol.* 68: 90-99; the phosphodiester method of Brown *et al.* (1979) *Meth. Enzymol.* 68: 109-151; the diethylphosphoramidite method of Beaucage *et al.* (1981) *Tetra. Lett.*, 22: 1859-1862; and the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This can be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is often limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Glycosyltransferase and accessory enzyme nucleic acids can be cloned using DNA amplification methods such as polymerase chain reaction (PCR). Thus, for example, the nucleic acid sequence or subsequence is PCR amplified, using a sense primer containing one restriction site (*e.g.*, *NdeI*) and an antisense primer containing another restriction site (*e.g.*, *HindIII*). This will produce a nucleic acid encoding the desired glycosyltransferase or accessory enzyme sequence or subsequence and having terminal restriction sites. This nucleic acid can then be easily ligated into a vector containing a nucleic acid encoding the second molecule and having the appropriate corresponding restriction sites. Suitable PCR primers can be determined by one of skill in the art using the sequence information provided in GenBank or other sources. Appropriate restriction sites can also be added to the nucleic acid encoding the glycosyltransferase protein or protein subsequence by site-directed mutagenesis. The plasmid containing the glycosyltransferase-encoding nucleotide sequence or subsequence is cleaved with the appropriate restriction endonuclease and then ligated into an appropriate vector for amplification and/or expression according to standard methods. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.*, eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3: 81-94; (Kwoh *et al.* (1989)

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Proc. Natl. Acad. Sci. USA 86: 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem.*, 35: 1826; Landegren *et al.*, (1988) *Science* 241: 1077-1080; Van Brunt (1990) *Biotechnology* 8: 291-294; Wu and Wallace (1989) *Gene* 4: 560; and Barringer *et al.* (1990) *Gene* 89: 117.

5 Other physical properties of a polypeptide expressed from a particular nucleic acid can be compared to properties of known glycosyltransferases or accessory enzymes to provide another method of identifying suitable nucleic acids. Alternatively, a putative glycosyltransferase or accessory enzyme gene can be mutated, and its role as a glycosyltransferase or accessory enzyme established by detecting a variation in the structure
10 of an oligosaccharide normally produced by the glycosyltransferase or accessory enzyme.

 In some embodiments, it may be desirable to modify the glycosyltransferase and/or accessory enzyme nucleic acids. One of skill will recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells
15 containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See, e.g.*, Gilman and Smith (1979) *Gene* 8:81-97, Roberts *et al.* (1987) *Nature* 328: 731-734.

 For example, the glycosyltransferase and/or accessory enzyme nucleic acids
20 can be modified to facilitate the linkage of the two domains to obtain the polynucleotides that encode the fusion polypeptides of the invention. Glycosyltransferase catalytic domains and accessory enzyme catalytic domains that are modified by such methods are also part of the invention. For example, codon for a cysteine residue can be placed at either end of a domain so that the domain can be linked by, for example, a sulfide linkage. The modification
25 can be done using either recombinant or chemical methods (*see, e.g.*, Pierce Chemical Co. catalog, Rockford IL). The glycosyltransferase and/or accessory enzyme catalytic domains are typically joined by linker domains, which are typically polypeptide sequences, such as poly glycine sequences of between about 5 and 200 amino acids, with between about 10-100 amino acids being typical. In some embodiments, proline residues are incorporated into the
30 linker to prevent the formation of significant secondary structural elements by the linker. Preferred linkers are often flexible amino acid subsequences which are synthesized as part of

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a recombinant fusion protein. In one embodiment, the flexible linker is an amino acid subsequence comprising a proline such as Gly(x)-Pro-Gly(x) where x is a number between about 3 and about 100. In other embodiments, a chemical linker is used to connect synthetically or recombinantly produced glycosyltransferase and accessory enzyme catalytic domains. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

In a preferred embodiment, the recombinant nucleic acids present in the cells of the invention are modified to provide preferred codons which enhance translation of the nucleic acid in a selected organism (e.g., yeast preferred codons are substituted into a coding nucleic acid for expression in yeast).

D. Expression cassettes and host cells for expressing the fusion polypeptides

Typically, the polynucleotide that encodes the fusion polypeptide is placed under the control of a promoter that is functional in the desired host cell. An extremely wide variety of promoters are well known, and can be used in the expression vectors of the invention, depending on the particular application. Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites and the like are also optionally included. Constructs that include one or more of these control sequences are termed "expression cassettes." Accordingly, the invention provides expression cassettes into which the nucleic acids that encode fusion polypeptides are incorporated for high level expression in a desired host cell.

Expression control sequences that are suitable for use in a particular host cell are often obtained by cloning a gene that is expressed in that cell. Commonly used prokaryotic control sequences, which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (*lac*) promoter systems (Change *et al.*, *Nature* (1977) 198: 1056), the tryptophan (*trp*) promoter system (Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8: 4057), the *tac* promoter (DeBoer, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1983) 80:21-25); and the lambda-derived P_L

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promoter and N-gene ribosome binding site (Shimatake *et al.*, *Nature* (1981) 292: 128). The particular promoter system is not critical to the invention, any available promoter that functions in prokaryotes can be used.

For expression of fusion polypeptides in prokaryotic cells other than *E. coli*, a
5 promoter that functions in the particular prokaryotic species is required. Such promoters can be obtained from genes that have been cloned from the species, or heterologous promoters can be used. For example, the hybrid *trp-lac* promoter functions in *Bacillus* in addition to *E. coli*.

A ribosome binding site (RBS) is conveniently included in the expression
10 cassettes of the invention. An RBS in *E. coli*, for example, consists of a nucleotide sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon (Shine and Dalgarno, *Nature* (1975) 254: 34; Steitz, *In Biological regulation and development: Gene expression* (ed. R.F. Goldberger), vol. 1, p. 349, 1979, Plenum Publishing, NY).

For expression of the fusion polypeptides in yeast, convenient promoters
15 include GAL1-10 (Johnson and Davies (1984) *Mol. Cell. Biol.* 4:1440-1448) ADH2 (Russell *et al.* (1983) *J. Biol. Chem.* 258:2674-2682), PHO5 (*EMBO J.* (1982) 6:675-680), and MF α (Herskowitz and Oshima (1982) in *The Molecular Biology of the Yeast Saccharomyces* (eds. Strathern, Jones, and Broach) Cold Spring Harbor Lab., Cold Spring Harbor, N.Y., pp. 181-209). Another suitable promoter for use in yeast is the ADH2/GAPDH hybrid promoter as
20 described in Cousens *et al.*, *Gene* 61:265-275 (1987). For filamentous fungi such as, for example, strains of the fungi *Aspergillus* (McKnight *et al.*, U.S. Patent No. 4,935,349), examples of useful promoters include those derived from *Aspergillus nidulans* glycolytic genes, such as the ADH3 promoter (McKnight *et al.*, *EMBO J.* 4: 2093-2099 (1985)) and the *tpiA* promoter. An example of a suitable terminator is the ADH3 terminator (McKnight *et al.*).
25

Suitable constitutive promoters for use in plants include, for example, the cauliflower mosaic virus (CaMV) 35S transcription initiation region and region VI promoters, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other promoters active in plant cells that are known to those of skill in the art. Other suitable
30 promoters include the full-length transcript promoter from Figwort mosaic virus, actin promoters, histone promoters, tubulin promoters, or the mannopine synthase promoter

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(MAS). Other constitutive plant promoters include various ubiquitin or polyubiquitin promoters derived from, *inter alia*, Arabidopsis (Sun and Callis, *Plant J.*, 11(5):1017-1027 (1997)), the mas, Mac or DoubleMac promoters (described in United States Patent No. 5,106,739 and by Comai *et al.*, *Plant Mol. Biol.* 15:373-381 (1990)) and other transcription initiation regions from various plant genes known to those of skill in the art. Such genes include for example, *ACT11* from Arabidopsis (Huang *et al.*, *Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from Arabidopsis (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding stearyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al.*, *Plant Physiol.* 104:1167-1176 (1994)), *Gpc1* from maize (GenBank No. X15596, Martinez *et al.*, *J. Mol. Biol.* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.* 33:97-112 (1997)). Useful promoters for plants also include those obtained from Ti- or Ri-plasmids, from plant cells, plant viruses or other hosts where the promoters are found to be functional in plants. Bacterial promoters that function in plants, and thus are suitable for use in the methods of the invention include the octopine synthetase promoter, the nopaline synthase promoter, and the manopine synthetase promoter. Suitable endogenous plant promoters include the ribulose-1,6-biphosphate (RUBP) carboxylase small subunit (ssu) promoter, the (α -conglycinin promoter, the phaseolin promoter, the ADH promoter, and heat-shock promoters.

Either constitutive or regulated promoters can be used in the present invention. Regulated promoters can be advantageous because the host cells can be grown to high densities before expression of the fusion polypeptides is induced. High level expression of heterologous proteins slows cell growth in some situations. An inducible promoter is a promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, temperature, pH, anaerobic or aerobic conditions, light, transcription factors and chemicals. Such promoters are referred to herein as "inducible" promoters, which allow one to control the timing of expression of the glycosyltransferase or enzyme involved in nucleotide sugar synthesis. For *E. coli* and other bacterial host cells, inducible promoters are known to those of skill in the art. These include, for example, the *lac* promoter, the bacteriophage lambda P_L promoter, the hybrid *trp-lac* promoter (Amann *et al.* (1983) *Gene* 25: 167; de Boer *et al.* (1983) *Proc. Nat'l. Acad. Sci.*

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USA 80: 21), and the bacteriophage T7 promoter (Studier *et al.* (1986) *J. Mol. Biol.*; Tabor *et al.* (1985) *Proc. Nat'l. Acad. Sci. USA* 82: 1074-8). These promoters and their use are discussed in Sambrook *et al.*, *supra*. A particularly preferred inducible promoter for expression in prokaryotes is a dual promoter that includes a *tac* promoter component linked to a promoter component obtained from a gene or genes that encode enzymes involved in galactose metabolism (e.g., a promoter from a UDPgalactose 4-epimerase gene (*galE*)). The dual *tac-gal* promoter, which is described in PCT Patent Application Publ. No. WO98/20111, provides a level of expression that is greater than that provided by either promoter alone.

10 Inducible promoters for use in plants are known to those of skill in the art (see, e.g., references cited in Kuhlemeier *et al.* (1987) *Ann. Rev. Plant Physiol.* 38:221), and include those of the 1,5-ribulose biphosphate carboxylase small subunit genes of *Arabidopsis thaliana* (the "ssu" promoter), which are light-inducible and active only in photosynthetic tissue, anther-specific promoters (EP 344029), and seed-specific promoters of, for example, *Arabidopsis thaliana* (Krebbers *et al.* (1988) *Plant Physiol.* 87:859).

Inducible promoters for other organisms are also well known to those of skill in the art. These include, for example, the arabinose promoter, the *lacZ* promoter, the metallothionein promoter, and the heat shock promoter, as well as many others.

A construct that includes a polynucleotide of interest operably linked to gene expression control signals that, when placed in an appropriate host cell, drive expression of the polynucleotide is termed an "expression cassette." Expression cassettes that encode the fusion polypeptides of the invention are often placed in expression vectors for introduction into the host cell. The vectors typically include, in addition to an expression cassette, a nucleic acid sequence that enables the vector to replicate independently in one or more selected host cells. Generally, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria. For instance, the origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria. Alternatively, the vector can replicate by becoming integrated into the host cell genomic complement and being replicated as the cell undergoes DNA replication. A preferred expression vector for expression of the enzymes is in bacterial cells

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is pTGK, which includes a dual *tac-gal* promoter and is described in PCT Patent Application Publ. NO. WO98/20111.

The construction of polynucleotide constructs generally requires the use of vectors able to replicate in bacteria. A plethora of kits are commercially available for the purification of plasmids from bacteria. For their proper use, follow the manufacturer's instructions (*see*, for example, EasyPrepJ, FlexiPrepJ, both from Pharmacia Biotech; StrataCleanJ, from Stratagene; and, QIAexpress Expression System, Qiagen). The isolated and purified plasmids can then be further manipulated to produce other plasmids, and used to transfect cells. Cloning in *Streptomyces* or *Bacillus* is also possible.

Selectable markers are often incorporated into the expression vectors used to express the polynucleotides of the invention. These genes can encode a gene product, such as a protein, necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics or other toxins, such as ampicillin, neomycin, kanamycin, chloramphenicol, or tetracycline. Alternatively, selectable markers may encode proteins that complement auxotrophic deficiencies or supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli. Often, the vector will have one selectable marker that is functional in, *e.g.*, *E. coli*, or other cells in which the vector is replicated prior to being introduced into the host cell. A number of selectable markers are known to those of skill in the art and are described for instance in Sambrook *et al.*, *supra*. A preferred selectable marker for use in bacterial cells is a kanamycin resistance marker (Vieira and Messing, *Gene* 19: 259 (1982)). Use of kanamycin selection is advantageous over, for example, ampicillin selection because ampicillin is quickly degraded by β -lactamase in culture medium, thus removing selective pressure and allowing the culture to become overgrown with cells that do not contain the vector.

Suitable selectable markers for use in mammalian cells include, for example, the dihydrofolate reductase gene (DHFR), the thymidine kinase gene (TK), or prokaryotic genes conferring drug resistance, *gpt* (xanthine-guanine phosphoribosyltransferase, which can be selected for with mycophenolic acid; *neo* (neomycin phosphotransferase), which can be selected for with G418, hygromycin, or puromycin; and DHFR (dihydrofolate reductase),

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which can be selected for with methotrexate (Mulligan & Berg (1981) *Proc. Nat'l. Acad. Sci. USA* 78: 2072; Southern & Berg (1982) *J. Mol. Appl. Genet.* 1: 327).

Selection markers for plant and/or other eukaryotic cells often confer resistance to a biocide or an antibiotic, such as, for example, kanamycin, G 418, bleomycin, hygromycin, or chloramphenicol, or herbicide resistance, such as resistance to chlorsulfuron or Basta. Examples of suitable coding sequences for selectable markers are: the *neo* gene which codes for the enzyme neomycin phosphotransferase which confers resistance to the antibiotic kanamycin (Beck *et al* (1982) *Gene* 19:327); the *hyg* gene, which codes for the enzyme hygromycin phosphotransferase and confers resistance to the antibiotic hygromycin (Gritz and Davies (1983) *Gene* 25:179); and the *bar* gene (EP 242236) that codes for phosphinothricin acetyl transferase which confers resistance to the herbicidal compounds phosphinothricin and bialaphos.

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques as described in the references cited above. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required. To confirm correct sequences in plasmids constructed, the plasmids can be analyzed by standard techniques such as by restriction endonuclease digestion, and/or sequencing according to known methods. Molecular cloning techniques to achieve these ends are known in the art. A wide variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant nucleic acids are well-known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Volume 152, Academic Press, Inc., San Diego, CA (Berger); and Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement) (Ausubel).

A variety of common vectors suitable for use as starting materials for constructing the expression vectors of the invention are well known in the art. For cloning in bacteria, common vectors include pBR322 derived vectors such as pBLUESCRIPT™, and λ-phage derived vectors. In yeast, vectors include Yeast Integrating plasmids (e.g., YIp5) and Yeast Replicating plasmids (the YRp series plasmids) and pGPD-2. Expression in

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mammalian cells can be achieved using a variety of commonly available plasmids, including pSV2, pBC12BI, and p91023, as well as lytic virus vectors (e.g., vaccinia virus, adeno virus, and baculovirus), episomal virus vectors (e.g., bovine papillomavirus), and retroviral vectors (e.g., murine retroviruses).

5 The methods for introducing the expression vectors into a chosen host cell are not particularly critical, and such methods are known to those of skill in the art. For example, the expression vectors can be introduced into prokaryotic cells, including *E. coli*, by calcium chloride transformation, and into eukaryotic cells by calcium phosphate treatment or electroporation. Other transformation methods are also suitable.

10 Translational coupling may be used to enhance expression. The strategy uses a short upstream open reading frame derived from a highly expressed gene native to the translational system, which is placed downstream of the promoter, and a ribosome binding site followed after a few amino acid codons by a termination codon. Just prior to the termination codon is a second ribosome binding site, and following the termination codon is
15 a start codon for the initiation of translation. The system dissolves secondary structure in the RNA, allowing for the efficient initiation of translation. See Squires, et. al. (1988), *J. Biol. Chem.* 263: 16297-16302.

 The fusion polypeptides can be expressed intracellularly, or can be secreted from the cell. Intracellular expression often results in high yields. If necessary, the amount
20 of soluble, active fusion polypeptide may be increased by performing refolding procedures (see, e.g., Sambrook *et al.*, *supra.*; Marston *et al.*, *Bio/Technology* (1984) 2: 800; Schoner *et al.*, *Bio/Technology* (1985) 3: 151). In embodiments in which the fusion polypeptides are secreted from the cell, either into the periplasm or into the extracellular medium, the DNA sequence is linked to a cleavable signal peptide sequence. The signal sequence directs
25 translocation of the fusion polypeptide through the cell membrane. An example of a suitable vector for use in *E. coli* that contains a promoter-signal sequence unit is pTA1529, which has the *E. coli* *phoA* promoter and signal sequence (see, e.g., Sambrook *et al.*, *supra.*; Oka *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) 82: 7212; Talmadge *et al.*, *Proc. Natl. Acad. Sci. USA* (1980) 77: 3988; Takahara *et al.*, *J. Biol. Chem.* (1985) 260: 2670).

30 The fusion polypeptides of the invention can also be further linked to other bacterial proteins. This approach often results in high yields, because normal prokaryotic

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control sequences direct transcription and translation. In *E. coli*, *lacZ* fusions are often used to express heterologous proteins. Suitable vectors are readily available, such as the pUR, pEX, and pMR100 series (see, e.g., Sambrook *et al.*, *supra.*). For certain applications, it may be desirable to cleave the non-glycosyltransferase and/or accessory enzyme amino acids from the fusion protein after purification. This can be accomplished by any of several methods known in the art, including cleavage by cyanogen bromide, a protease, or by Factor X_a (see, e.g., Sambrook *et al.*, *supra.*; Itakura *et al.*, *Science* (1977) 198: 1056; Goeddel *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) 76: 106; Nagai *et al.*, *Nature* (1984) 309: 810; Sung *et al.*, *Proc. Natl. Acad. Sci. USA* (1986) 83: 561). Cleavage sites can be engineered into the gene for the fusion protein at the desired point of cleavage.

More than one fusion polypeptide may be expressed in a single host cell by placing multiple transcriptional cassettes in a single expression vector, or by utilizing different selectable markers for each of the expression vectors which are employed in the cloning strategy.

A suitable system for obtaining recombinant proteins from *E. coli* which maintains the integrity of their N-termini has been described by Miller *et al.* *Biotechnology* 7:698-704 (1989). In this system, the gene of interest is produced as a C-terminal fusion to the first 76 residues of the yeast ubiquitin gene containing a peptidase cleavage site. Cleavage at the junction of the two moieties results in production of a protein having an intact authentic N-terminal residue.

Fusion polypeptides of the invention can be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The host cells can be mammalian cells, plant cells, or microorganisms, such as, for example, yeast cells, bacterial cells, or fungal cells. Examples of suitable host cells include, for example, *Azotobacter sp.* (e.g., *A. vinelandii*), *Pseudomonas sp.*, *Rhizobium sp.*, *Erwinia sp.*, *Escherichia sp.* (e.g., *E. coli*), *Bacillus*, *Pseudomonas*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Rhizobia*, *Vitreoscilla*, *Paracoccus* and *Klebsiella sp.*, among many others. The cells can be of any of several genera, including *Saccharomyces* (e.g., *S. cerevisiae*), *Candida* (e.g., *C. utilis*, *C. parapsilosis*, *C. krusei*, *C. versatilis*, *C. lipolytica*, *C. zeylanoides*, *C. guilliermondii*, *C. albicans*, and *C. humicola*), *Pichia* (e.g., *P. farinosa* and *P. ohmeri*), *Torulopsis* (e.g., *T.*

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candida, *T. sphaerica*, *T. xylinus*, *T. famata*, and *T. versatilis*), Debaryomyces (e.g., *D. subglobosus*, *D. cantarellii*, *D. globosus*, *D. hansenii*, and *D. japonicus*), Zygosaccharomyces (e.g., *Z. rouxii* and *Z. bailii*), Kluyveromyces (e.g., *K. marxianus*), Hansenula (e.g., *H. anomala* and *H. jadinii*), and Brettanomyces (e.g., *B. lambicus* and *B. anomalus*). Examples of useful bacteria include, but are not limited to, *Escherichia*,
5 *Enterobacter*, *Azotobacter*, *Erwinia*, *Klebsiella*,.

The expression vectors of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by
10 the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the *amp*, *gpt*, *neo* and *hyg* genes.

In preferred embodiments, fusion polypeptides that comprise eukaryotic glycosyltransferase and accessory enzyme catalytic domains are expressed in eukaryotic host cells. Similarly, fusion polypeptides that comprise prokaryotic catalytic domains are
15 preferably expressed in prokaryotic cells. Alternatively, one can express a mammalian fusion polypeptide in a prokaryotic host cell (see, e.g., Fang et al. (1998) *J. Am. Chem. Soc.* 120: 6635-6638), or vice versa.

Once expressed, the recombinant fusion polypeptides can be purified according to standard procedures of the art, including ammonium sulfate precipitation,
20 affinity columns, column chromatography, gel electrophoresis and the like (see, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Protein Purification*, Academic Press, Inc. N.Y. (1990)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred. Once purified, partially or to
25 homogeneity as desired, the polypeptides may then be used (e.g., as immunogens for antibody production).

To facilitate purification of the fusion polypeptides of the invention, the nucleic acids that encode the fusion polypeptides can also include a coding sequence for an epitope or "tag" for which an affinity binding reagent is available. Examples of suitable
30 epitopes include the myc and V-5 reporter genes; expression vectors useful for recombinant production of fusion polypeptides having these epitopes are commercially available (e.g.,

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Invitrogen (Carlsbad CA) vectors pcDNA3.1/Myc-His and pcDNA3.1/V5-His are suitable for expression in mammalian cells). Additional expression vectors suitable for attaching a tag to the fusion proteins of the invention, and corresponding detection systems are known to those of skill in the art, and several are commercially available (e.g., FLAG" (Kodak, Rochester NY). Another example of a suitable tag is a polyhistidine sequence, which is capable of binding to metal chelate affinity ligands. Typically, six adjacent histidines are used, although one can use more or less than six. Suitable metal chelate affinity ligands that can serve as the binding moiety for a polyhistidine tag include nitrilo-tri-acetic acid (NTA) (Hochuli, E. (1990) "Purification of recombinant proteins with metal chelating adsorbents" In Genetic Engineering: Principles and Methods, J.K. Setlow, Ed., Plenum Press, NY; commercially available from Qiagen (Santa Clarita, CA)).

Other haptens that are suitable for use as tags are known to those of skill in the art and are described, for example, in the Handbook of Fluorescent Probes and Research Chemicals (6th Ed., Molecular Probes, Inc., Eugene OR). For example, dinitrophenol (DNP), digoxigenin, barbiturates (see, e.g., US Patent No. 5,414,085), and several types of fluorophores are useful as haptens, as are derivatives of these compounds. Kits are commercially available for linking haptens and other moieties to proteins and other molecules. For example, where the hapten includes a thiol, a heterobifunctional linker such as SMCC can be used to attach the tag to lysine residues present on the capture reagent.

One of skill would recognize that modifications can be made to the glycosyltransferase and accessory enzyme catalytic domains without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the catalytic domain into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, the addition of codons at either terminus of the polynucleotide that encodes the catalytic domain to provide, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

E. Uses of the fusion polypeptides

The invention provides methods of using fusion polypeptides produced using the methods described herein to prepare desired oligosaccharides (which are composed of

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two or more saccharides). The glycosyltransferase reactions of the invention take place in a reaction medium comprising at least one glycosyltransferase, an acceptor sugar and typically a soluble divalent metal cation. Substrates for the accessory enzyme catalytic moiety are also present, so that the accessory enzyme can synthesize the donor moiety for the

5 glycosyltransferase. The methods rely on the use of a glycosyltransferase to catalyze the addition of a saccharide to a substrate saccharide. For example, the invention provides methods for adding sialic acid to a galactose residue in an α 2,3 linkage, by contacting a reaction mixture that includes an acceptor moiety comprising a Gal residue in the presence of an α 2,3-sialyltransferase/CMP-NeuAc synthetase fusion polypeptide that has been

10 prepared according to the methods described herein. The reaction mixture also includes sialic acid and CTP, as well as other ingredients necessary for activity of the sialyltransferase and the CMP-NeuAc synthetase.

A number of methods of using glycosyltransferases to synthesize desired oligosaccharide structures are known. Exemplary methods are described, for instance, WO

15 96/32491, Ito *et al.* (1993) *Pure Appl. Chem.* 65: 753, and US Patents 5, 352,670, 5,374,541, and 5,545,553.

The fusion polypeptides prepared as described herein can be used in combination with additional glycosyltransferases. For example, one can use a combination of sialyltransferase fusion polypeptide and a galactosyltransferase, which may or may not be

20 part of a fusion polypeptide. In this group of embodiments, the enzymes and substrates can be combined in an initial reaction mixture, or preferably the enzymes and reagents for a second glycosyltransferase reaction can be added to the reaction medium once the first glycosyltransferase reaction has neared completion. By conducting two glycosyltransferase reactions in sequence in a single vessel, overall yields are improved over procedures in

25 which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

The products produced by the above processes can be used without purification. However, it is usually preferred to recover the product. Standard, well known techniques for recovery of glycosylated saccharides such as thin or thick layer

30 chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a nanofiltration or reverse

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osmotic membrane as described in commonly assigned US Patent Application Ser. No. 08/947,775, filed October 9, 1997. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 1000 to about 10,000 can be used to remove proteins. Nanofiltration or reverse osmosis can then be used to remove salts. Nanofilter membranes are a class of reverse osmosis membranes which pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 200 to about 1000 Daltons, depending upon the membrane used. Thus, in a typical application, the oligosaccharides of the invention will be retained in the membrane and contaminating salts will pass through.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

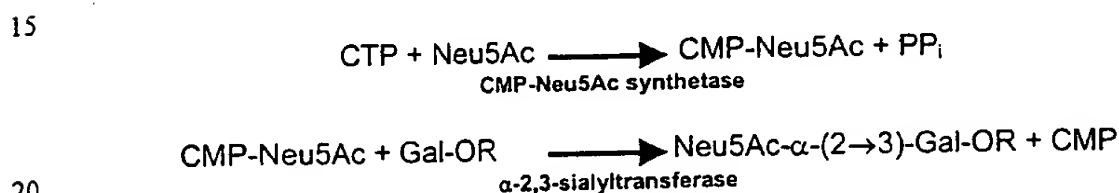
Example 1

Construction of a CMP-Neu5Ac Synthetase/ α 2,3-Sialyltransferase Fusion Protein

This Example describes the construction and expression of a polynucleotide that encodes a fusion protein that has both CMP-Neu5Ac synthetase activity and α 2,3-sialyltransferase activity. Large-scale enzymatic synthesis of oligosaccharides containing terminal *N*-acetyl-neuraminic acid residues requires large amounts of the sialyltransferase and the corresponding sugar-nucleotide synthetase for the synthesis of the sugar-nucleotide donor, CMP-Neu5Ac, an unstable compound. Using genes cloned from *Neisseria meningitidis*, we constructed a fusion protein which has both CMP-Neu5Ac synthetase and α -2,3-sialyltransferase activities. The fusion protein was produced in high yields (over 1,200 units per liter, measured using an α -2,3-sialyltransferase assay) in *Escherichia coli* and functionally pure enzyme could be obtained using a simple protocol. In small-scale enzymatic syntheses, we showed that the fusion protein could sialylate various oligosaccharide acceptors (branched and linear) with *N*-acetyl-neuraminic acid as well as *N*-glycolyl- and *N*-propionyl-neuraminic acid in high conversion yield. The fusion protein was also used to produce α -2,3-sialyllactose at the 100 g scale using a sugar nucleotide cycle reaction, starting from lactose, sialic acid, phosphoenolpyruvate and catalytic amounts of ATP and CMP.

Previously we reported the cloning and over-expression in *Escherichia coli* of both the CMP-Neu5Ac synthetase (Gilbert *et al.* (1997) *Biotechnol. Lett.* 19: 417-420) and the α -2,3-sialyltransferase (Gilbert *et al.* (1996) *J. Biol. Chem.* 271: 28271-28276; Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194) from *Neisseria meningitidis*. The two enzymes
 5 were used together to synthesize milligram amounts of sialyllactose, sialyl-N-acetyllactosamine and sialyl-P^k (Neu5Ac- α -(2 \rightarrow 3)-Gal- α -(1 \rightarrow 4)-Gal- β -(1 \rightarrow 4)-Glc). The CMP-Neu5Ac synthetase can also be used to produce CMP derivatives of sialic acid analogs in order to synthesize the corresponding sialo-oligosaccharide analogs (*Id.*).

Although we obtained a high yield (750 U/L) of the α -2,3-sialyltransferase in
 10 *E. coli* (*Id.*), the purified enzyme was relatively insoluble and had a tendency to precipitate and lose activity during storage. Since the CMP-Neu5Ac synthetase was necessary for synthesis purposes and was a soluble enzyme, we decided to make a fused form of these two enzymes to see if it would be more soluble than the individual α -2,3-sialyltransferase. The following two reactions would therefore be catalyzed by the same polypeptide:



The fused form of these enzymes would also be kinetically favorable since the CMP-Neu5Ac synthetase has a turnover number (Gilbert *et al.* (1997) *Biotechnol. Lett.* 19: 417-420) of 31.4 sec⁻¹ while the α -2,3-sialyltransferase has turnover numbers ranging
 25 from 0.1 to 1.4 sec⁻¹, depending on the acceptor (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194 and unpublished data). The fused form would have the additional benefit of reducing enzyme production costs by having a single culture to grow and a single product to purify to obtain the two activities.

Materials and Methods

30 *Construction of the fusion CMP-Neu5Ac synthetase/ α -2,3-sialyltransferase.*

PCR was performed with *Pwo* polymerase as described by the manufacturer (Boehringer Mannheim, Laval, Que.). The *Neisseria* CMP-Neu5Ac synthetase was

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amplified using SYNTM-F1 as the 5' primer (41 mer: 5'-CTTAGGAGGTCATATGGAA
AAACAAAATATTGCGGTTATAC-3' (SEQ ID NO: 3); the *NdeI* site is in italics) and
SYNTM-R6 as the 3' primer (45-mer: 5'-CGACAGAAATCCGCCACCGCTTTCCTT
GTGATTAAGAATGTTTTC-3' (SEQ ID NO: 4); the *EcoRI* site is in italics) and pNSY-01
5 (Gilbert *et al.* (1997) *Biotechnol. Lett.* 19: 417-420) as the template.

The *Neisseria* α -2,3-sialyltransferase was amplified using SIALM-22F as the 5'
primer (37-mer: 5'-GCATGGAATTCTGGGCTTGAAAAAGGCTTGTTTGACC-3' (SEQ ID
NO: 5); the *EcoRI* site is in italics) and SIALM-23R as the 3' primer (59-mer: 5'-
CCTAGGTCGACTCATTAGTGGTGATGGTG GTGATGGTTCAGGTCTTCTTCG-
10 CTGATCAG-3' (SEQ ID NO: 6); the *SaII* site is in italics, the 6-His tail is underlined and the
c-myc tag is in bold) and using pNST-09 (Gilbert *et al.* (1996) *J. Biol. Chem.* 271: 28271-
28276) as the template. The plasmid pFUS-01 was constructed by digesting the CMP-
Neu5Ac synthetase PCR product with *NdeI* and *EcoRI* and the α -2,3-sialyltransferase PCR
product with *EcoRI* and *SaII* and cloning them in a modified version of pCWori+ (Gilbert *et*
15 *al.* (1997) *Eur. J. Biochem.* 249: 187-194), in which the *lacZ α* gene fragment has been
deleted.

Expression in E. coli and purification of the fusion protein

The initial screening of pFUS-01 versions was done using *E. coli* BMH71-18
as the host. For the large-scale production of the fusion protein we used *E. coli* AD202
20 (CGSC #7297). A 21 L culture of *E. coli* AD202/pFUS-01/2 was grown in a 28-L New
Brunswick Scientific (Edison, NJ) fermenter (model MF 128S) as described previously
(Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194). The cells were resuspended in 50 mM
Hepes pH 7 at a ratio of 20 g of wet cell paste for 80 mL of buffer. Cell extracts were
prepared using an Avestin C5 Emulsiflex cell disrupter (Avestin, Ottawa, Ont.).
25 Polyethylene glycol (average molecular weight 8,000 Da) and NaCl were added to 4% and
0.2 M, respectively, and the cell extract was stirred 20 min at 4 °C. The extract was
centrifuged 20 min at 8000 rpm and the pellet was washed twice with 50 mM Hepes pH 7,
0.2 M NaCl, 4% PEG. The pellet was resuspended with 50 mM Tris, pH 7.5, 1 mM EDTA
and Triton X-100 (reduced and peroxide-free) was added to 1% v/v. The resuspended pellet
30 was stirred 30 min at 4 °C and then clarified by centrifugation for 1 h at 13,000 x g. The
supernatant was applied to two 5-mL HiTrap Chelating column (Pharmacia Biotech, Uppsala,

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Sweden) charged with Ni^{2+} , the maximum load being 25 mg total protein in each run. The columns were developed with a 60-800 mM imidazole gradient in 10 mM Hepes (pH 7) containing 0.5 M NaCl and 0.2% Triton X-100.

Assays

5 Protein concentration was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL). For all of the enzymatic assays one unit of activity was defined as the amount of enzyme that generated one μmol of product per minute. The CMP-Neu5Ac synthetase activity was assayed at 37 °C using 3 mM Neu5Ac, 3 mM CTP, 100 mM Tris pH 8.5, 0.2 mM DTT and 10 mM MgCl_2 in a final volume of 50 μL . The reaction was
10 stopped after 10 min by adding EDTA to 20 mM final concentration and the reaction mixture was analyzed by capillary electrophoresis performed with a Beckman Instruments (Fullerton, CA) P/ACE 5510 equipped with a P/ACE diode array detector set at 271 nm and using the separation conditions described previously (Gilbert *et al.* (1997) *Biotechnol. Lett.* 19: 417-420).

15 All acceptors were synthesized as previously described (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194; Wakarchuk *et al.* (1996) *J. Biol. Chem.* 271: 19166-19173) with the exception that FEX (# F-6130, Molecular Probes, Eugene, OR) was used in place of FCHASE for the LacNAc acceptor.

The α -2,3-sialyltransferase activity was assayed at 37 °C using 0.5 mM
20 LacNAc-FEX, 0.2 mM CMP-Neu5Ac, 50 mM Mes pH 6.0, 10 mM MnCl_2 in a final volume of 10 μL . After 5 min the reactions were terminated by dilution with 10 mM NaOH and analyzed by capillary electrophoresis performed using the separation conditions described previously (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194).

The coupled assay was performed using similar conditions except that the
25 incubation time was 10 min and the reaction mixture included 0.5 mM LacNAc-FEX, 3 mM CTP, 3 mM Neu5Ac, 100 mM Tris pH 7.5, 0.2 mM DTT and 10 mM MgCl_2 . The same reagent concentrations were used when the alternate acceptors (Lac-FCHASE and P^k -FCHASE) or the alternate donors (Neu5Gc and Neu5Pr) were tested, except the reaction times were 60 to 120 min.

30 Sialylation of a biantennary acceptor was performed using 1 mg of Gal- β -(1 \rightarrow 4)-GlcNAc- β -(1 \rightarrow 2)-Man- α -(1 \rightarrow 6)-[Gal- β -(1 \rightarrow 4)-GlcNAc- β -(1 \rightarrow 2)-Man- α -(1 \rightarrow 3)]-

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Man- β -(1 \rightarrow 4)-GlcNAc- β -(1 \rightarrow 4)-GlcNAc in a 90 min reaction. Reaction progress was monitored by TLC using isopropanol/H₂O/ammonium hydroxide (6:3:1) to develop the plate and the sialylated product was purified by gel filtration on Bio-Gel P-4 (Bio-Rad Lab., Hercules, CA). The mass of the isolated compound was determined by mass spectrometry
5 (negative ion mode).

Use in a 100 g scale synthesis

The reaction was performed in a total volume of 2.2 L and the following reagents were added sequentially: lactose monohydrate (59.4 g, 0.165 mol), phosphoenolpyruvate monopotassium salt (34 g, 0.165 mol), bovine serum albumin (2.2g), sialic acid
10 (51 g, 0.165 mol), CMP (2.84 g, 8.79 mmol), ATP (0.532 g, 0.879 mmol) and sodium azide (0.44 g). The pH was adjusted to 7.4 with NaOH and MnCl₂ was added to a final concentration of 30 mM. The reaction was allowed to proceed at room temperature after the addition of 13,200 units of myokinase (Boehringer Mannheim), 19,800 units of pyruvate kinase (Boehringer Mannheim) and 820 units (based on α -2,3-sialyltransferase activity) of
15 fusion protein obtained by extraction with Triton X-100 of the PEG/NaCl precipitate. Reaction progress was monitored daily by TLC using isopropanol/H₂O/ammonium hydroxide (7:2:1) to develop the plate and orcinol/sulfuric acid followed by heating to visualize the product. Mn²⁺ was monitored daily by ion chromatography and the reaction mixture was supplemented with 1M MnCl₂ to maintain a final concentration of 30 mM.
20 Supplementary phosphoenolpyruvate was added after two days (0.165 mol) and four days (0.055 mol).

After a total reaction time of 6 days, the crude α -2,3-sialyllactose solution was filtered through two sheets of Whatman filter paper to remove the precipitate producing a yellow filtrate. Proteins were then removed by tangential flow ultrafiltration using a
25 3,000 MWCO membrane (#P2PLBCC01, Millipore, Bedford, MA), providing a clear yellow solution. Triton X-100 was removed from the reaction mixture by filtration through a column containing 500 g of C18 reverse phase resin. The eluate was then further purified using a nanofiltration machine (#19T-SSXYC-PES-316-SP, Osmonics, Minnetonka, MN) fitted with a spiral wound membrane (#GE2540C1076) and using two different pH's. The pH
30 of the solution was first adjusted with concentrated HCl to pH = 3.0, and the feed solution was recirculated for 10 hours while maintaining the total volume of the feed by continuous

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addition of deionized water. When the conductivity of the permeate solution reached 22 mS, the pH was adjusted to pH = 7.0 with 50% NaOH. Recirculation of this solution while maintaining the feed volume with deionized water was performed for an additional 2 hours. The feed solution was concentrated to 800 mL and was then treated with AG50WX8 (H+) Dowex resin until a pH of 2.0 was reached. After removing the resin by filtration, the pH was adjusted to 7.0 with NaOH and the solution was decolorized by passing through activated charcoal. The solution was finally lyophilized to yield a white powder and the α -2,3-sialyllactose content was determined by ^1H NMR analysis in D_2O using 1,2-isopropylidene- α -D-glucofuranose as the reference standard.

10 Results

Construction of the fusion CMP-Neu5Ac synthetase/ α -2,3-sialyltransferase

The *Neisseria* CMP-Neu5Ac synthetase was amplified by PCR, using primers that included a *NdeI* site (5') and an *EcoRI* site (3'), while the *Neisseria* α -2,3-sialyltransferase was amplified using primers that included an *EcoRI* site (5') and a *SalI* site (3'). The two PCR products were cloned together in a modified version of pCWori+ (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194) that was digested with *NdeI* and *SalI*. In the resulting construct (pFUS-01) the start codon of the CMP-Neu5Ac synthetase was downstream of the three sequential IPTG-inducible promoters and the ribosome binding site present in pCWori+. The α -2,3-sialyltransferase was linked to the C-terminal of the CMP-Neu5Ac synthetase through a 4-residue peptide linker (Gly-Gly-Gly-Ile) and the C-terminus of the fusion protein includes a c-Myc epitope tag for immuno-detection and a His₆ tail for purification by immobilized metal affinity chromatography (IMAC). In the process of cloning pFUS-01 we also obtained 2 clones that included additional residues in the linker regions. The linker of pFUS-01/2 (see Figure 1) is 9 residues long (Gly-Gly-Gly-Ile-Leu-Ser-His-Gly-Ile; SEQ ID NO: 7) while the linker of pFUS-01/4 is 8 residues long (Gly-Gly-Gly-Ile-Leu-Ser-Gly-Ile; SEQ ID NO: 8). Analysis by DNA sequencing of the two versions with additional residues suggested that they were cloning artifacts due to incomplete restriction enzyme digestion of the PCR products.

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Expression in E. coli and purification of the fusion protein.

E. coli BMH71-18 was transformed with the three versions of pFUS-01 and the level of α -2,3-sialyltransferase activity was compared in small-scale cultures (20 mL). The highest activity was obtained with pFUS-01/2, which gave 40% more activity than pFUS-01/4 and 60% more activity than pFUS-01. The fusion protein encoded by pFUS-01/2 has the longest linker which might aid the independent folding of the two components. However, the effects of linker composition and length were not further studied and pFUS-01/2 was used for the scale-up in production and kinetics comparison.

Since we had observed an OmpT-catalyzed degradation when pFUS-01/2 was expressed in *E. coli* BMH71-18 (data not shown) we used an *ompT*-deficient host strain (*E. coli* AD202) for expression. In a 21 L culture of *E. coli* AD202/pFUS-01/2, we measured a production of 1,200 U per liter using an assay for α -2,3-sialyltransferase activity, 11,500 U per liter using an assay for CMP-Neu5Ac synthetase activity and 300 U per liter using a coupled CMP-Neu5Ac synthetase/ α -2,3-sialyltransferase assay. SDS-PAGE analysis indicated that a band with the expected molecular mass (70.2 kDa) of the fusion enzyme was predominant in the extract. The activity was associated with the insoluble fraction of the extract since over 95% of the activity was recovered in the pellet when the extract was centrifuged at 100,000 x g for 1 hour. This situation was similar to what we observed with the separate α -2,3-sialyltransferase when it was over-expressed in *E. coli* (*Id.*). The α -2,3-sialyltransferase is membrane bound in *N. meningitidis* (Gilbert *et al.* (1996) *J. Biol. Chem.* 271: 28271-28276) and it is not surprising that, when over-expressed separately or as a fusion protein in *E. coli*, part of it was associated with the membranes and/or cell debris.

In order to avoid large-scale ultracentrifugation, we developed a precipitation strategy to recover the activity associated with the insoluble fraction at a lower centrifugation speed (12,000 x g). Precipitation with 4% polyethylene glycol (PEG 8000) and 0.2 M NaCl afforded over 95% recovery of activity in the pellet, with a 1.8 fold increase in specific activity between the crude extract (0.32 U/mg) and the PEG/NaCl precipitate (0.58 U/mg). The pellet was washed with buffer containing PEG/NaCl in order to remove traces of soluble (cytosolic) enzymes such as hydrolases that could degrade essential cofactors and substrates used in the enzymatic synthesis of target oligosaccharides. Although the washing steps reduced slightly the enzyme recovery, it was essential to obtain functionally pure fusion protein.

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The PEG/NaCl precipitate was extracted with 1% Triton X-100 in order to solubilize the activity. We recovered 60-70 % of the enzyme activity in the soluble fraction which represented a 40-55 % yield when compared with the activity present in the total extract and a 3 fold increase in specific activity (1 U/mg). The material extracted with Triton X-100 from the PEG/NaCl precipitate was stable for at least a month at 4 °C and was used in the synthesis reactions described below.

Immobilized metal affinity chromatography (IMAC) was performed on the Triton X-100 extract and the fusion protein appeared in the fractions eluting between 400 and 550 mM imidazole. The purified fusion protein had a specific activity of 1-2 U/mg and the overall purification yield was below 5%. Analysis of the purified protein by SDS-PAGE showed that it was at least 90% pure.

Comparison of the fusion protein with the individual enzymes

This comparison was made difficult by the fact that the enzymes differ widely in their solubility and tendency to aggregate when purified to homogeneity. We observed previously that the CMP-Neu5Ac synthetase was soluble to above 20 mg/mL (Gilbert *et al.* (1997) *Biotechnol. Lett.* 19: 417-420) while the α -2,3-sialyltransferase precipitated when attempts were made to concentrate it above 1 mg/mL, even in the presence of detergent (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194). The IMAC-purified fusion protein was soluble to about 5 mg/mL in the presence of 0.2 % Triton X-100. Using the α -2,3-sialyltransferase assay we found specific activities in the range of 1 to 1.5 U/mg for different batches of the purified separate α -2,3-sialyltransferase and 1 to 2 U/mg for different batches of the purified fusion protein. A tendency to aggregate might explain the relatively large variation in specific activity between different batches of IMAC-purified fusion protein.

Previously we observed that partially purified α -2,3-sialyltransferase could be extracted with Triton X-100 from membrane fractions obtained by ultracentrifugation (*Id.*). This procedure is similar to the extraction of the fusion protein from the PEG/NaCl precipitate but the extraction from the membranes yielded purer material. Such preparations of both the fusion protein and the separate α -2,3-sialyltransferase were more stable than the IMAC-purified material, but since the enzyme was not homogeneous the protein concentration was estimated by scanning densitometry of SDS-PAGE gels. Using this procedure we observed a specific activity of 2.0 U/mg for the separate α -2,3-

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sialyltransferase and 2.7 U/mg for the fusion protein. When taking into account the molecular masses of these two proteins, we calculated turnover numbers of 1.4 sec⁻¹ for the separate α -2,3-sialyltransferase and 3.2 sec⁻¹ for the fusion enzyme. Given the different solubility properties of these two proteins, it is difficult to conclude if there is any real catalytic improvement of the α -2,3-sialyltransferase when it is in the fused form or if it is simply more stable under the assay conditions. On the other hand, the CMP-Neu5Ac synthetase turnover number of the fused form was comparable to the turnover number of the separate CMP-Neu5Ac synthetase (39.5 sec⁻¹ and 31.4 sec⁻¹, respectively).

Small scale syntheses with various donors and acceptors

The ability of the fusion protein to use different donors and acceptors was tested in analytical (5 nmol) coupled reactions performed at pH 7.5 which is intermediate between the optimal pH of the α -2,3-sialyltransferase (pH 6) (Gilbert *et al.* (1996) *J. Biol. Chem.* 271: 28271-28276) and the optimal pH of the CMP-Neu5Ac synthetase (pH 8.5) (Warren and Blacklow (1962) *J. Biol. Chem.* 237: 3527-3534). The fusion protein could sialylate *N*-acetylglucosamine-FEX and lactose-FCHASE with *N*-acetyl-neuraminic acid as well as the *N*-propionyl- and *N*-glycolyl- analogs in yields that exceeded 97% in 1 hour (Table 1). Both *N*-acetyl-glucosamine-FEX and lactose-FCHASE have a terminal β -Gal which is the natural acceptor for the *Neisseria* α -2,3-sialyltransferase (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194).

Table 1: Small-scale syntheses using the fusion CMP-Neu5Ac synthetase/ α -2,3-sialyltransferase with various donors and acceptors (% conversion to sialylated product).

Acceptor	Donor ^a		
	Neu5Ac	Neu5Pr	Neu5Gc
Gal- β -(1 \rightarrow 4)-GlcNAc- β ^b (60 min reaction)	>99	>99	>99
Gal- β -(1 \rightarrow 4)-Glc- β ^c (60 min reaction)	>99	97	97
Gal- α -(1 \rightarrow 4)-Gal- β -(1 \rightarrow 4)- β -Glc- β ^c (120 min reaction)	84	84	55

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Acceptor	Donor ^a		
	Neu5Ac	Neu5Pr	Neu5Gc
Biantennary N-linked type ^d (90 min reaction)	>99	ND ^e	ND

^a Neu5Ac = *N*-acetyl-neuraminic acid

Neu5Pr = *N*-propionyl-neuraminic acid

Neu5Gc = *N*-glycolyl-neuraminic acid

5

^b This acceptor was a FEX-aminophenyl-glycoside derivative.

^c These acceptors were FCHASE-aminophenyl-glycosides derivatives.

10 ^d Gal-β-(1→4)-GlcNAc-β-(1→2)-Man-α-(1→6)\
Man-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc
Gal-β-(1→4)-GlcNAc-β-(1→2)-Man-α-(1→3)/

^e Not determined.

15

When P^k-FCHASE (Gal-α-(1→4)-Gal-β-(1→4)-Glc-FCHASE) was used as the acceptor in 2 hour reactions, the sialylation yield was 84% with either *N*-acetyl- or *N*-propionyl-neuraminic acid while it was 55% with *N*-glycolyl-neuraminic acid (Table 1). We had observed previously that P^k-FCHASE was a substrate for the α-2,3-sialyltransferase but it was found to have a k_{cat}/K_m 4 to 40-fold lower than substrates which have terminal β-Gal (Gilbert *et al.* (1997) *Eur. J. Biochem.* 249: 187-194). *N*-glycolyl-neuraminic acid gave the lowest sialylation yields with the three acceptors tested, which is not surprising since the *Neisseria* CMP-Neu5Ac synthetase had a K_m that was 8-fold higher with *N*-glycolyl-neuraminic acid than with *N*-acetyl-neuraminic acid (Gilbert *et al.* (1997) *Biotechnol. Lett.* 19: 417-420).

25

The fusion protein can also use branched oligosaccharides as acceptors since we observed >99% sialylation of an asialo- galactosylated biantennary N-linked type oligosaccharide using *N*-acetyl-neuraminic acid as the donor (Table 1). This reaction was done at the 1 mg scale using the underivatized oligosaccharide and the mass of the isolated

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product (2224.0 Da) was found to agree with the mass of the expected di-sialylated biantennary oligosaccharide (2223.3 Da).

Use in a 100 g scale synthesis

The material extracted with Triton X-100 from the PEG/NaCl precipitate was used in a 100 g scale synthesis to produce α -2,3-sialyllactose using the sialyltransferase cycle (Ichikawa *et al.* (1991) *J. Am. Chem. Soc.* 113: 4698-4700) starting from lactose, sialic acid, phosphoenolpyruvate (PEP), and catalytic amounts of ATP and CMP. After 6 days of reaction, the reaction had reached completion as evidenced by the disappearance of sialic acid by TLC analysis. The product was then purified by a sequence of ultrafiltration, nanofiltration and ion exchange. This process yielded 77 g of a white solid which had an α -2,3-sialyllactose content of 88 % and a water content of 7 %. Based on the α -2,3-sialyllactose content of the isolated product, the overall yield for the synthesis and isolation was 68%.

Discussion

The CMP-Neu5Ac synthetase/ α -2,3-sialyltransferase fusion protein was expressed at high level in a cost-effective expression system and showed both enzyme activities at levels comparable to those of the individual enzymes. It was readily recoverable by a simple protocol involving precipitation and detergent extraction, therefore avoiding expensive chromatographic steps. The detergent extracted fusion protein was functionally pure, *i.e.* it was free from contaminating enzyme activities that can hydrolyze sugar nucleotides or other components of the cofactor regeneration system.

To be useful for large scale carbohydrate synthesis the fusion protein should be applicable in a sugar nucleotide cycle. This cycle is designed to use only catalytic amounts of expensive sugar nucleotides and nucleoside phosphates, which are enzymatically regenerated *in situ* from low-cost precursors. The recycling of the converted co-factors also prevents end-product inhibition. The α -2,3-sialyllactose 100 g scale synthesis went to completion, which is important since stoichiometric conversion of substrates is desirable not only to minimize reagent costs but also because it greatly simplifies the purification of the product from a large scale synthesis. Another interesting feature of the fusion protein is that it can use directly different donor analogs and various acceptors with a terminal galactose

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residue. Consequently it can be used for the synthesis of both natural carbohydrates and synthetic derivatives with novel properties.

The CMP-Neu5Ac synthetase/ α -2,3-sialyltransferase fusion protein was expressed in high yield in *E. coli* with the two components being at least as active as the separate enzymes, which indicates that they were folded properly. This example suggests that construction and expression of fusion proteins may be of general utility to produce the enzymes required for large-scale biotechnological processes involving multiple enzymatic steps.

Example 2

Construction of a UDP-Glucose Epimerase / β -1,4-Galactosyltransferase Fusion Protein

The use of sugar nucleotide cycling systems (SNC) oligosaccharide synthesis requires a number of enzymes. The purification of these enzymes is a time consuming and expensive part of the process. In the first example we produced a fusion protein which combines a transferase with its corresponding sugar-nucleotide synthetase (FUS-01), and have shown the advantages of a simple purification of the two activities. In this example we have produced a fusion of two other proteins used in SNC reactions, the UDP-Glucose 4 epimerase (*galE*) and a β -1,4-galactosyltransferase (*lgtB*).

Materials and Methods

DNA manipulations

The *S. thermophilus* UDP-glucose 4' epimerase (*galE*) gene was amplified from pTGK-EP1 using primers derived from the nucleotide sequence of *galE* from *Streptococcus thermophilus* (GenBank accession M38175). GalE-5p was used as the 5' primer (58 mer: 5'-GGGACAGGATCCATCGATGCTTAGGAGGTCATATGGCAATT TAGTATTAGGTGGAGC-3' (SEQ ID NO: 9); the *Bam*HI site is in bold and italics)(primers used in this Example are shown in Figure 4) and GalE-3p as the 3' primer (42-mer: 5'-GGGGGGGCTAGCGCCGCCTCCTCGATCATCG TACCCTTTTGG-3' (SEQ ID NO: 10); the *Nhe*I site is in italics). The plasmid pTGK/EP1, which includes the *galE* gene was used (see, PCT Patent Application Publ. No. WO98/20111) as the template.

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The *Neisseria* β -1,4-galactosyltransferase was amplified using LgtB-NheI as the 5' primer (38-mer: 5'-GGGGGGGCTAGCGTGCAAAACCACGTTATCAGCTTAGC-3' (SEQ ID NO: 11); the *NheI* site is in italics) and LgtB-SaII as the 3' primer (45-mer: 5'-GGGGGGGTCGACCTATTATTGGAAAGGCACAATGAACTGTTCGCG-3' (SEQ ID NO: 12); the *SaII* site is in italics) and using pCW-lgtB(MC58) (Wakarchuk *et al.* (1998) *Protein Engineering* 11: 295-302) as the template. The thermocycler parameters were 94 °C 3 min., and 30 cycles of 55°C 30 sec., 72°C 30 sec., 94°C 30 sec. PCR was performed with Pwo polymerase as described by the manufacturer (Boehringer Mannheim, Laval, Que.). The nucleotide (SEQ ID NO: 13) and deduced amino acid (SEQ ID NO: 14) sequences of the *Neisseria* β -1,4-galactosyltransferase are shown in Figure 2.

The plasmid pFUS-EB was constructed as follows (Figure 3). The UDP-glucose 4 epimerase PCR product was digested with *Bam*HI and *NheI* and the β -1,4-galactosyltransferase PCR product was digested with *NheI* and *SaII* and then recovered from the reaction mixtures using Prep-a-Gene™ resin according to the manufacturer's instruction (BioRad). The two genes were then combined in a three fragment ligation under standard conditions with the vector pCWori⁺ (Wakarchuk *et al.* (1994) *Protein Science* 3: 467-475) that had been digested with *Bam*HI and *SaII*. DNA was introduced into *E. coli* DH12S using electroporation with 1 μ l of the ligation reaction. Transformants were screened using colony PCR with primers specific for vector sequences flanking the cloning site. Colonies with inserts of the correct size, were then grown in liquid culture and tested for enzyme activity.

Determination of Enzyme activity

Standard reactions for the β -1,4-galactosyltransferase enzyme were performed at 37°C in 20 μ l of: HEPES-NaOH buffer 50 mM, pH 7.5 containing, 10 mM MnCl₂, 1.0 mM fluorescein labeled acceptor, 1.0 mM UDP-Gal donor and various amounts of enzyme extract from recombinant *E. coli* that contains the cloned gene. The preparation of the fluorescein labeled acceptors was as described in Wakarchuk *et al.* (1996) *J. Biol. Chem.* 271 (32): 19166-19173 and Wakarchuk *et al.* (1998) *Protein Engineering* 11: 295-302.

Reactions to assess the epimerase-transferase fusion protein were performed with 1.0 mM UDP-Glucose in place of UDP-Gal. Enzymes were assayed after dilution of extracts in buffer containing 1 mg/ml acetylated bovine serum albumin. For calculation of enzyme activity, the enzyme dilutions were chosen such that for reaction times of 5-15

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minutes approximately 10% conversion of the acceptor to product would be achieved. The reactions were terminated either by the addition of an equal volume of 2% SDS and heated to 75°C, for 3 minutes, or by diluting the reaction with 10 mM NaOH. These samples were then diluted appropriately in water prior to analysis by capillary electrophoresis (Wakarchuk *et al.* (1996) *supra.*).

Small scale extracts were made as follows. The cells were pelleted in an 1.5 ml microcentrifuge tube 2 min. at maximum speed, and the medium discarded. The pellet was frozen and then mixed with 2 volumes of 150 μ m glass beads (Sigma), and ground with a glass pestle in the microcentrifuge tube. This mixture was then extracted twice with 50 μ l of 50 mM HEPES-NaOH pH 7.5. The supernatant from this was used as the source of material for enzyme assays. Larger scale extractions and the PEG-8000 precipitation were performed as described in Gilbert *et al.* (1998) *Nature Biotechnology* 16: 769- 772.

To verify that the product from reactions with the epimerase-transferase fusion using UDP-Glc was Gal- β -1,4-GlcNac-aminophenyl-FEX (FEX-LacNac), reaction products were separated by TLC and then eluted in methanol. After drying under vacuum, the samples were dissolved in water and glycosidase assays were performed as described in Wakarchuk *et al.* (1996), *supra.* These samples were then analyzed by TLC against standards of the FEX-LacNac and the degradation product, FEX-GlcNac (data not shown).

Results

The pFUS-EB construct was investigated for its induction kinetics. The fusion protein was inducible, but the enzyme activity accumulates to its highest level in shake flasks without any IPTG being added. Activity of the fusion protein was measured with either UDP-Gal or UDP-Glc as the donor. Assays performed using FEX-GlcNac as an acceptor show the amount of transferase activity using UDP-Glc as the donor is similar to the amount of transferase activity using UDP-Gal as the donor. The level of expression is such that from 1 L of shakeflask culture between 130-200 U of are produced.

With the CMP-NANA/ α -2,3-sialyltransferase fusion protein, we have shown the utility of concentrating the enzyme with PEG-8000/NaCl precipitations (Example 1). We have investigated using PEG-8000/NaCl for recovery of the β -1,4-galactosyltransferase fusion/UDP-glucose 4 epimerase fusion polypeptide from the cell free extracts. Since it appears to be a very soluble protein, we used 16% PEG-8000, which is a higher level than

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we had used for the other fusion protein. We did not see any adverse affects on enzyme activity after the PEG-8000 recovery step. It appears that the protein is not inhibited by the PEG precipitation step, and that recovery of active protein is high. It also appears that when the activity is measured in samples with higher concentrations of enzyme, using pre-formed
5 UDP-Gal, that the activity is lower. This may be because the epimerase converts some of the UDP-Gal back to UDP-Glc, which makes the activity appear lower.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be
10 suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

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WHAT IS CLAIMED IS:

- 1 1. A nucleic acid which comprises a polynucleotide that encodes a fusion
2 polypeptide, wherein the fusion polypeptide comprises:
3 a) a catalytic domain of a glycosyltransferase; and
4 b) a catalytic domain of an accessory enzyme which catalyzes a step in
5 the formation of a nucleotide sugar which is a saccharide donor for the glycosyltransferase.
- 1 2. The nucleic acid of claim 1, wherein the glycosyltransferase is a
2 eukaryotic glycosyltransferase.
- 1 3. The nucleic acid of claim 1, wherein the accessory enzyme is a
2 eukaryotic accessory enzyme.
- 1 4. The method of claim 2, wherein the catalytic domain of the
2 glycosyltransferase substantially lacks one or more of a cytoplasmic domain, a signal-anchor
3 domain, and a stem region of the glycosyltransferase.
- 1 5. The nucleic acid of claim 1, wherein the glycosyltransferase is a
2 prokaryotic glycosyltransferase.
- 1 6. The nucleic acid of claim 1, wherein the accessory enzyme is a
2 prokaryotic accessory enzyme.
- 1 7. The nucleic acid of claim 1, wherein the fusion polypeptide further
2 comprises a catalytic domain of a second accessory enzyme.
- 1 8. The nucleic acid of claim 1, wherein the glycosyltransferase is selected
2 from the group consisting of sialyltransferases, *N*-acetylglucosaminyltransferases, *N*-
3 acetylgalactosaminyltransferases, fucosyltransferases, galactosyltransferases,
4 glucosyltransferases, glucuronosyltransferases, xylosyltransferases, and
5 mannosyltransferases.

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1 9. The nucleic acid of claim 1, wherein the accessory enzyme is selected
2 from the group consisting of:
3 a GDP-mannose dehydratase, a GDP-mannose 3,5-epimerase, and a
4 GDP-mannose 4-reductase;
5 a UDP-glucose 4' epimerase;
6 a UDP-GalNAc 4' epimerase;
7 a CMP-sialic acid synthetase;
8 a neuraminic acid aldolase;
9 an *N*-acetylglucosamine 2' epimerase;
10 a phosphate kinase selected from the group consisting of a pyruvate
11 kinase, a myokinase, a creatine phosphate kinase, an acetyl phosphate kinase, and a
12 polyphosphate kinase; and
13 a pyrophosphorylase selected from the group consisting of a UDP-Glc
14 pyrophosphorylase, a UDP-Gal pyrophosphorylase, a UDP-GalNAc pyrophosphorylase, a
15 GDP-mannose pyrophosphorylase, a GDP-fucose pyrophosphorylase, and a UDP-GlcNAc
16 pyrophosphorylase.

1 10. The nucleic acid of claim 1, wherein the nucleotide sugar is selected
2 from the group consisting of GDP-Man, UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc,
3 CMP-sialic acid, GDP-Fuc, and UDP-xylose.

1 11. The nucleic acid of claim 1, wherein the glycosyltransferase is a
2 sialyltransferase and the nucleotide sugar is CMP-sialic acid.

1 12. The nucleic acid of claim 11, wherein the accessory enzyme is a CMP-
2 sialic acid synthetase.

1 13. The nucleic acid of claim 11, wherein the accessory enzyme is a
2 neuraminic acid aldolase or an *N*-acetylglucosamine 2' epimerase.

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1 14. The nucleic acid of claim 1, wherein the glycosyltransferase is a
2 galactosyltransferase and the nucleotide sugar is UDP-galactose.

1 15. The nucleic acid of claim 14, wherein the accessory enzyme is a UDP-
2 glucose 4' epimerase.

1 16. The nucleic acid of claim 1, wherein the glycosyltransferase is a
2 fucosyltransferase and the nucleotide sugar is GDP-fucose.

1 17. The nucleic acid of claim 16, wherein the accessory enzyme is selected
2 from the group consisting of a GDP-mannose dehydratase, a GDP-mannose 3,5-epimerase, a
3 GDP-fucose pyrophosphorylase, and a GDP-mannose 4-reductase.

1 18. The nucleic acid of claim 1, wherein the glycosyltransferase is an *N*-
2 acetylgalactosaminyltransferase and the nucleotide sugar is UDP-GalNAc.

1 19. The nucleic acid of claim 18, wherein the accessory enzyme is a UDP-
2 GalNAc 4' epimerase.

1 20. The nucleic acid of claim 1, wherein the glycosyltransferase is an *N*-
2 acetylglucosaminyltransferase and the nucleotide sugar is UDP-GlcNAc.

1 21. The nucleic acid of claim 20, wherein the accessory enzyme is a UDP-
2 GalNAc 4' epimerase.

1 22. The nucleic acid of claim 1, wherein the glycosyltransferase is a
2 mannosyltransferase and the nucleotide sugar is GDP-Man.

1 23. The nucleic acid of claim 1, wherein the fusion polypeptide further
2 comprises a linker peptide between the glycosyltransferase catalytic domain and the
3 accessory enzyme catalytic domain.

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1 24. The nucleic acid of claim 1, wherein the nucleic acid further comprises
2 a polynucleotide that encodes a signal sequence which is linked to the fusion polypeptide.

1 25. The nucleic acid of claim 1, wherein the nucleic acid further comprises
2 a polynucleotide that encodes a molecular tag which is linked to the fusion polypeptide.

1 26. An expression vector which comprises a nucleic acid of claim 1.

1 27. A host cell which comprises a nucleic acid of claim 1.

1 28. A fusion polypeptide encoded by a nucleic acid of claim 1.

1 29. A fusion polypeptide that comprises:
2 a) a catalytic domain of a glycosyltransferase; and
3 b) a catalytic domain of an accessory enzyme which catalyzes a step in
4 the formation of a nucleotide sugar which is a donor for the glycosyltransferase.

1 30. The fusion polypeptide of claim 29, wherein the catalytic domain of the
2 glycosyltransferase is joined to the carboxy terminus of the accessory enzyme catalytic
3 domain.

1 31. The fusion polypeptide of claim 29, wherein the glycosyltransferase is a
2 galactosyltransferase and the accessory enzyme is a UDP-glucose 4' epimerase.

1 32. The fusion polypeptide of claim 29, wherein the glycosyltransferase is a
2 sialyltransferase and the accessory enzyme is a CMP-sialic acid synthetase.

1 33. A method of producing a fusion polypeptide that comprises:
2 a) a catalytic domain of a glycosyltransferase; and
3 b) a catalytic domain of an accessory enzyme which catalyzes a step in
4 the formation of a nucleotide sugar which is a donor for the glycosyltransferase;

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5 wherein the method comprises introducing a nucleic acid that encodes
6 the fusion polypeptide into a host cell to produce a transformed host cell; and culturing the
7 transformed host cell under conditions appropriate for expressing the fusion polypeptide.

1 34. The method of claim 33, wherein the fusion polypeptide is purified
2 following its expression.

1 35. The method of claim 33, wherein the host cell is permeabilized
2 following expression of the fusion polypeptide.

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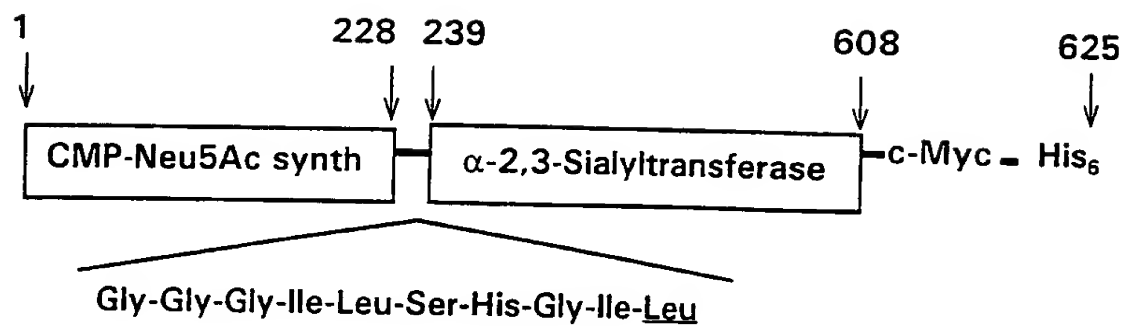


Fig. 1

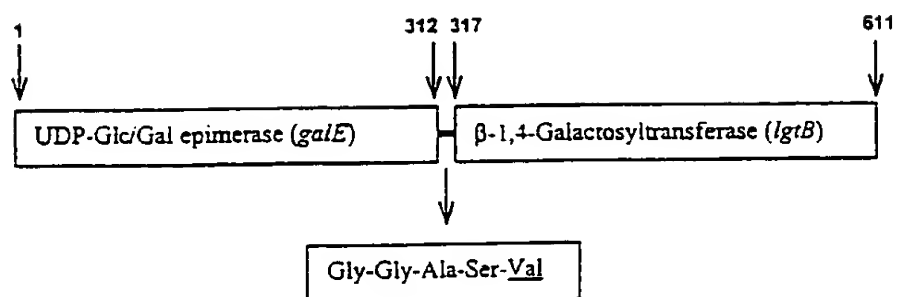
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Figure 3



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Figure 4

A. 5' primer for amplification of *galE* for insertion into pCW at the BamHI site

5' GGGACAGGATCCATCGATGCTTAGGAGGTCATATGGCAATTTTAGTATTAGGTGGAGC 3'
 BamHI Met

B. 3' primer for amplification of *galE* for fusion with *lgtB* insertion into pCW

5' GGGGGGGCTAGCGCCGCTCCTCGATCATCGTACCCTTTTGG 3'
 NheI Gly Gly

C. 5' primer for amplification of *lgtB* for fusion with the 3'-end of *galE*.

5' GGGGGGGCTAGCGTGCAAAACCACGTTATCAGCTTAGC
 NheI Val

D. 3' primer for amplification of *lgtB* for fusion with *galE* and insertion into pCW

5' GGGGGGGTCGACCTATTATTGGAAAGGCACAATGAACTGTTTCGCG
 SalI

E. Junction region of the *galE-lgtB* fusion

										<i>galE</i>						NheI						<i>lgtB</i>					
5'	CCA	AAA	GGG	TAC	GAT	GAT	CGA	GGA	GGC	GGA	GCT	AGC	GTG	CAA	AAC	CAC	GTT	ATC	AGC	TTA	GCT	3'					
3'	GGT	TTT	CCC	ATG	CTA	CTA	GCT	CCT	CCG	CCT	CGA	TCG	CAC	GTT	TTG	GTG	CAA	TAG	TCG	AAT	CGA	5'					
	P	K	G	Y	D	D	R	G	G	G	A	S	V	Q	N	H	V	I	S	L	A						

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INTERNATIONAL SEARCH REPORT

In International Application No
PCT/CA 98/01180

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/10 C12N15/52 C12N15/62 C12N15/74 C12N15/79
C12P19/02 C12P19/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. GILBERT ET AL., : "Purification and characterization of the recombinant CMP-sialic acid synthetase from <i>Neisseria meningitidis</i> " BIOTECHNOLOGY LETTERS, vol. 19, no. 5, May 1997, pages 417-420, XP002105242 cited in the application see the whole document and specially page 420. --- -/--	1,5,6

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/01180

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. GILBERT ET AL., : "Characterization of a recombinant Neisseria meningitidis alpha-2,3-sialyltransferase and its receptor acceptor specificity" EUROPEAN JOURNAL OF BIOCHEMISTRY , vol. 249, no. 1, 1997, pages 187-194, XP002105243 cited in the application see discussion and specially page 193 ---	1-3,8,10
A	WO 96 21038 A (ALKO GROUP LTD ;MARAS MARLEEN (BE); CONTRERAS ROLAND (BE)) 11 July 1996 see page 3, paragraph 3 - page 4, last paragraph see page 19, last paragraph see page 20, paragraph 3 - page 23, paragraph 2; examples 5,69,10,11 ---	1-3, 8-12,14, 18,20
A	WO 96 32491 A (CYTEL CORP) 17 October 1996 cited in the application see page 3, line 8-19 see page 11, line 37 - page 12, line 36 see page 15, line 12 - page 16, line 26; example 2 ---	1,8-12, 14,16
A	WO 96 35801 A (HOECHST AG ;HOERSCH BRIGITTE (DE); SEIFFERT STOERIKO ANDREAS (DE);) 14 November 1996 see page 1, paragraph 1; examples 7,11 ---	1,8-10, 14,15
A	WO 96 40971 A (NEOSE TECHNOLOGIES INC) 19 December 1996 see page 6, line 20 - page 12, line 20 see page 19 - page 21 ---	1,8,10
P,A	WO 98 07837 A (AUSTIN RESEARCH INST ;MCKENZIE IAN FARQUHAR CAMPBELL (AU); SANDRIN) 26 February 1998 see page 2, line 29 - page 3, line 11 see page 3, line 24 - page 7, line 9 ---	1,5,6,8
P,X	M. GILBERT ET AL., : "The synthesis of sialylated oligosaccharides using a CMP-Neu5Ac synthetase/sialyltransferase fusion" NATURE BIOTECHNOLOGY, vol. 16, no. 8, August 1998, pages 769-772, XP002105244 cited in the application see the whole document -----	1-3, 8-12, 23-30, 32-35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 98/01180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9621038 A	11-07-1996	US 5834251 A AU 4348696 A EP 0799318 A	10-11-1998 24-07-1996 08-10-1997
WO 9632491 A	17-10-1996	US 5728554 A AU 5387396 A CA 2218246 A EP 0820519 A JP 11503329 T	17-03-1998 30-10-1996 17-10-1996 28-01-1998 26-03-1999
WO 9635801 A	14-11-1996	DE 19516952 A CA 2220675 A EP 0827549 A	14-11-1996 14-11-1996 11-03-1998
WO 9640971 A	19-12-1996	AU 5965596 A CA 2224144 A EP 0832277 A	30-12-1996 19-12-1996 01-04-1998
WO 9807837 A	26-02-1998	AU 3841897 A	06-03-1998

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